NOVEL SALICYLIC ACID-BINDING PROTEIN ENCODING NUCLEIC ACID, SABP2, AND METHODS OF USE THEREOF

Daniel F. Klessig
and Dhirendra Kumar

This application is a continuation in part of international application PCT/US02/26312, filed August 16, 2002, which in turn claims priority to US provisional Application, 60/312,863 filed August 16, 2001, the entire contents of which are incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Science Foundation, Grant No. MCB-0196046, IBN-0110272, and IBN-0241531.

FIELD OF THE INVENTION

This invention relates to proteins involved in signal transduction pathways in higher plants. More specifically, the invention provides a novel nucleic acid molecule encoding a protein involved in stress and disease resistance pathways in multicellular plants. The biological molecules of the invention may be used to advantage to (i) create transgenic plants, (ii) identify compounds that induce or enhance disease resistance in plants, and (iii) elucidate the molecular mechanisms responsible for modulation of salicylic acid-mediated disease resistance in plants.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by author name and year of publication in

parentheses in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. Several patent documents are also referenced to facilitate the practice of the present invention. The disclosure of each of these publications and patent documents is incorporated by reference herein.

Plants can respond to infection by microbial pathogens through the activation of a variety of defense responses. At the sites of infection, a hypersensitive response (HR) is often initiated. The hallmark of this response is the formation of necrotic lesions, a process that is likely due to programmed host cell death. In addition, associated with the HR is the restriction of growth and spread of pathogens. Frequently, defense responses are also activated in tissues distal to the sites of infection according to a phenomenon known as systemic acquired resistance (SAR). Development of SAR results in an enhanced and long-lasting resistance to secondary challenge by the same or even unrelated pathogens. Associated both with HR (and associated local resistance) and with SAR is the expression of pathogenesis-related (PR) genes, several of whose products have been shown to have antimicrobial activity (for review, see Klessig and Malamy, 1994; Hammond-Kosach and Jones, 1996; Durner et al., 1997; Dempsey et al., 1999).

A mounting body of evidence indicates that salicylic acid (SA) plays a key role in the activation of certain defense responses in a number of dicotyledonous species. For example, rises in endogenous SA levels correlate with the induction of PR genes and development of resistance in tobacco and cucumber (Malamy et al., 1990 and 1992, Métraux et al., 1990; Rasmussen et al., 1991). In

addition, several mutants of *Arabidopsis* (e.g., cpr's, lsd's, acd's) have been isolated which constitutively express PR genes and show enhanced resistance. They also demonstrate elevated levels of SA (Bowling et al., 1994; Dietrich et al., 1994; Greenberg et al., 1994; Yoshioka et al., 2001). Conversely, *Arabidopsis* mutants defective in SA signal transduction (e.g., npr1, nim1, sai1) exhibit enhanced susceptibility to pathogens (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Exogenously applied SA also induces PR gene expression and enhanced resistance in tobacco (White, 1979; Antoniw and White, 1980) and a variety of other plants (for review, see Klessig and Malamy, 1994; Durner et al., 1997). Furthermore, transgenic *Arabidopsis* and tobacco plants that express the bacterial salicylate hydroxylase (nahG) gene, whose product converts SA into biologically inactive catechol, fail to develop SAR and show increased susceptibility to primary infections by both virulent and avirulent pathogens (Gaffney et al., 1993; Delaney et al., 1994).

During the past several years, attempts to elucidate the mechanisms of SA action in plant disease resistance have been made by identifying the cellular components with which SA interacts. Initial studies led to the identification of a SA-binding protein, SABP, that was later shown to be a catalase (CAT) (Chen and Klessig, 1991; Chen et al., 1993a). Further analysis demonstrated that SA inhibited tobacco catalase activity in suspension cells and in crude leaf extracts. SA also inhibited the purified enzyme (Chen et al., 1993b; Conrath et al., 1995; Durner and Klessig, 1996). Thus, it was proposed that increases in SA after pathogen infection might inhibit catalase activity, producing elevated levels of H_2O_2 that could activate certain defense responses,

including PR gene expression. Supporting this hypothesis was the observation that prooxidants induced PR-1 gene expression (Chen et al., 1993b), while antioxidants suppressed the SA-mediated expression of PR-1 genes (Conrath et al., 1995; Wendehenne et al., 1998). In addition, the other major H_2O_2-scavenging enzyme, ascorbate peroxidase (APX), was subsequently shown to be inhibited by SA (Durner and Klessig, 1995).

In contrast, several other studies have questioned the role of H_2O_2 and the SA-mediated inhibition of CAT and APX during the activation of defense responses. No detectable increases in H_2O_2 levels were found during the establishment of SAR (Neuenschwander et al., 1995) and significant reductions in CAT activity were not observed in tobacco infected with *Pseudomonas syringae* or in leaf discs pretreated with SA (Bi et al., 1995). In addition, H_2O_2 and H_2O_2-inducing chemicals were unable to induce PR-1 gene expression in NahG transgenic plants (Bi et al., 1995; Neuenschwander et al., 1995). Moreover, high concentrations of H_2O_2 (150 mM - 1000 mM) were shown to induce SA accumulation (Neuenschwander et al., 1995; León et al., 1995; Summermatter et al., 1995). Finally, transgenic plants having significantly lower CAT activity via transformation with CAT antisense or cosuppressing sense constructs, did not exhibit constitutive PR-1 gene expression unless there was concurrent development of necrosis (Chamnonpol et al., 1996; Takahashi et al., 1997). From these results, it appears that H_2O_2 acts upstream of SA in the signal transduction cascade rather than, or in addition to, acting downstream of SA.

One possible explanation for these conflicting findings is that SA and H_2O_2 are involved in a self-amplifying feedback loop (Van Camp, 1998; Draper, 1997; Shirasu et al., 1997). An alternative mechanism through

which SA-mediated inhibition of CAT may signal defense responses is via the generation of SA free radicals, which could then activate a lipid peroxidation pathway (Durner and Klessig, 1996, Anderson et al., 1998). However, this possibility remains a topic of debate (Tenhaken and Rübel, 1997).

Taken together, these studies suggest that the activation of defense responses is mediated through the interaction of SA with other cellular factors, rather than, or in addition to interactions with CAT and APX. Thus, the isolation and identification of additional cellular factors associated with SA action in plant disease resistance is essential for elucidating the molecular mechanisms directing SA-mediated defense responses.

SUMMARY OF THE INVENTION

In accordance with the present invention, a novel nucleic acid molecule, *SABP2*, is provided. *SABP2* encodes the novel high-affinity salicylic acid-binding protein 2 (SABP2) which is involved in the SA-mediated signal transduction pathway leading to defense responses to pathogen infection in higher plants.

In one aspect of the invention, an isolated nucleic acid molecule comprising an SABP2-encoding nucleic acid sequence is provided. In a preferred embodiment, the nucleic acid molecule is isolated from a plant and has the SABP2-encoding nucleic acid sequence of SEQ ID NO: 1. The nucleic acid molecules of the invention may be inserted into an expression vector, such as a plasmid or viral vector, and may further be transformed into a host cell or plants. Exemplary host cells and plants include tobacco, *Arabidopsis*, rice, maize, wheat, tomato, potato, barley, canola, bacteria, yeast, insect and mammalian

cells.

According to another aspect of the invention, an isolated SABP2 polypeptide encoded by the nucleic acid sequence of SEQ ID NO: 1 is provided. In a preferred embodiment, the SABP2 polypeptide comprises the amino acid sequence of SEQ ID NOS: 2 and has enzymatic activity. In another embodiment of the invention, antibodies immunologically specific for at least one epitope of an SABP2 polypeptide are also provided.

According to yet another aspect of the invention, methods for identifying agents which modulate SABP2 function are provided. The methods comprise introducing SABP2-encoding nucleic acid molecules into a host cell, treating the cells or resulting plant with agents suspected of modulating SABP2 function and assaying SABP2 function in the presence and absence of the agent. The agents may modulate one or more of SABP2 expression, SABP2 binding affinity, or SABP2 enzymatic activity.

According to a preferred aspect of the invention, a method is provided to enhance resistance of a plant to plant pathogens or other disease causing agents comprising overexpressing a SABP2-encoding nucleic acid molecule having the sequence of SEQ ID NO: 1. In another embodiment of the invention, SABP2 expression is enhanced by the addition of an agent that enhances SABP2 expression in a host cell.

According to yet another aspect of the invention, a method is provided to inhibit SABP2 function in a plant. This method comprises introducing a mutated SABP2-encoding nucleic acid molecule into a plant which encodes a non-functional SABP2 protein. In a preferred embodiment, the SABP2-encoding nucleic acid molecule is an antisense molecule of SEQ ID NO: 1. Alternatively, methods for inhibition of SABP2 expression comprise

introduction of nucleic acid constructs effective to cause dsRNA mediated gene silencing of the SABP2 gene (Smith et al., 2000; Wesley et al., 2001).

According to another aspect of the present invention, transgenic plants comprising SEQ ID NO: 1 are provided. The transgenic plants of the invention exhibit enhanced disease resistance. Knock-out plants wherein SABP2 expression has been eliminated are also provided. Also within the scope of the invention are transgenic plants comprising variants of SEQ ID NO: 1 encoding proteins having altered SABP2 function.

In a further embodiment of the invention, the screening methods provided above are performed employing nucleic acids, proteins and antibodies encoded by the SABP2 homologs and functional orthologs identified in Arabidopsis (e.g., AtSB2L5) and other species. GenBank Accession numbers of exemplary nucleic acid sequences are provided in Table IV. These functionally equivalent homologs to SABP2 may also be used to modulate disease resistance in higher plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the resolution of the protein composition from pooled protein fractions containing the highest levels of SA-binding activity from each step in the purification of SABP2 on a 12.5% SDS-polyacrylamide gel. The right lane shows the resolution of an aliquot of fraction #16 of the second Mono Q column which contained the highest amount of SA-binding activity. The molecular size markers are in the left-most lane.

Figure 2 shows a graph depicting the elution profile of SA-binding activity from the Superdex G75 HR 10/30 gel filtration FPLC column. The position of the molecular

mass markers in kiloDaltons are indicated by the arrows. The SA-binding activity of each fraction was determined using [^{3}H-SA].

Figure 3 shows the resolution of three proteins (28-32 kDa) on a 12.5% SDS-polyacrylamide gel that co-purify with SA-binding activity. The molecular size markers are in lane M. Lanes 14-19 are fractions eluted from the second mono Q column. The relative SA-binding activity is given in arbitrary units above each fraction.

Figure 4 shows the nucleotide and deduced amino acid sequences of a SABP2 clone isolated from leaves of tobacco (SEQ ID NOS: 1 and 2). The five peptide sequences obtained by microsequencing are underlined in the deduced SABP2 amino acid sequence.

Figure 5 shows the amino acid sequence homology of SABP2 to hydroxynitrile lyases and eukaryotic esterases. Aligned from top to bottom are the deduced amino acid sequence of SABP2 (SEQ ID NO: 2), alpha-hydroxynitrile lyase (S-acetone-cyanohydrin lyase) from *Manihot esculenta* (SEQ ID NO: 3), S-acetone-cyanohydrin lyase from *Hevea brasiliensis* (SEQ ID NO: 4), a putative S-acetone-cyanohydrin lyase from *Arabidopsis thaliana* (SEQ ID NO: 5) and an esterase from rice, PIR7B (SEQ ID NO: 6). The residues of the catalytic triad are indicated by triangles and the lipase signature sequence of SABP2 is boxed.

Figures 6A and 6B show that SABP2 has lipase activity which is stimulated by salicylic acid. Figure 6A shows an in gel lipase assay of purified recombinant His6-SABP2 fractionated on an SDS-PAGE and then reacted

with the synthetic lipase/esterase substrate, MUF butyrate. The fluorescence at the position of SABP2 in the gel is due to the release of MUF by SABP2's lipase/esterase activity. Figure 6B is a graph which shows that SABP2 lipase activity is stimulated in the presence of salicylic acid. Lipase assays were performed with purified recombinant SABP2 (rSABP2) in either 50 mM bicine, pH 8.0 or 50 mM Tris-Cl, pH 8.0 containing 0.05% Triton-X100 and 1 mM lipase substrate. Results from assays using para-nitrophenol palmitate (pNPP) as the substrate in the presence or absence of SA are presented as an average with three separate preparations of recombinant SABP2, while those using pNP myristate (pNPM) as the substrate were done with one preparation of rSABP2. Lipase activity detected in each sample is presented in relative units and the fold stimulation by SA is shown in parentheses above the bars. One relative unit is the amount of enzyme that releases 0.017 mol/min of p-nitrophenol. Note that when 50 mM bicine is replaced with 50 mM Tris-HCl pH 8.0, SABP2 does not bind SA.

Figures 7A and 7B show blots of SABP2 induction at the mRNA [7A] and protein [7B] levels by TMV infection of resistant Xanthi nc [NN], but not susceptible Xanthi [nn], Nicotiana tabacum. Antibody against SABP2 was used in the immunoblot shown in Figure 7B.

Figures 8A-8G show that silencing of SABP2 suppresses disease resistance (as measured by tobacco mosaic virus (TMV) lesion size, and level of coat protein (CP) mRNA) and also suppresses PR-1 gene induction by SA and by TMV. Figure 8A - SABP2 expression was monitored by RT-PCR using beta-tubulin as an internal control. Figure 8B - 24 hours post treatment with SA; induction of

PR-1 expression with SA and TMV was monitored by northern (RNA blot) analysis, using EtBr stained rRNA as control for equal loading (not shown). Figure 8C - TMV-induced lesion size in mm. Figure 8D - Analysis of 36 hours post infection time point confirmed suppression of PR-1 induction by TMV in SABP2-silenced lines (data not shown). Figure 8E shows RNA blot analysis of TMV CP and SABP2 transcript accumulation and size of TMV induced lesion in control plants (transformed with empty vector) and various SABP2-silenced lines from the T_2 generation. Total RNA was isolated from TMV-inoculated leaves harvested at the indicated time points. Following transfer, the membrane was hybridized with probes for the SABP2 and TMV CP. The size of TMV-induced lesions on these control and SABP2-silenced lines was determined by measuring an average of 50 lesions per line. Lesion diameter is presented +/- standard deviation. Figure 8F shows a comparison of SA-induced PR-1 expression and SABP2 silencing in the T_1 generations of control and SABP2-silenced lines. Total RNA was isolated from all lines at 0 and 24 h post treatment (hpt) with 0.25 mM SA. PR-1 expression was monitored by RNA blot analysis while SABP2 expression was determined by RT-PCR analysis using cDNA generated from untreated plants. The level of EF1α product was monitored as an internal control to normalize the amount of cDNA template. Please note that PR-1 induction by SA was tested at least twice in two independent experiments for both the T_1 and T_2 generations to confirm the difference in SA responsiveness. Figure 8G shows a comparison of SA-induced PR-1 expression and SABP2 silencing in three different T_2 generation plants for line # 1. See (Figure 8F) above for details.

Figures 9A-C show that silencing SABP2 expression

blocks development of systemic acquired resistance (SAR). Figure 9A shows the size of primary lesions developed by TMV-inoculated control and SABP2-silenced plants (T_2 generation), measured at 7 dpi. Seven days after the primary TMV infection, the upper previously uninoculated leaves received a secondary inoculation with TMV. The diameter of the secondary lesions was then measured 7 days after the challenge infection. Each value represents the average size (in mm +/- standard deviation) of 50 lesions per line. Figure 9B shows morphology of TMV-induced lesions in control and SABP2-silenced tobacco. The leaves were photographed 7 days after a secondary infection with TMV. Figure 9C shows RNA blot analysis of TMV movement protein (MP) and PR-1 transcript accumulation in control and SABP2-silenced T_2 lines. Total RNA was isolated from systemic leaves prior to (0 day) or 7 days after a secondary inoculation with TMV (*). Following transfer, the membrane was hybridized with probes for the TMV MP and PR-1.

Figure 10 shows the relationship of Arabidopsis thaliana SABP2-like (AtSB2L) proteins to each other and to tobacco SABP2 (TSABP2)

DETAILED DESCRIPTION OF THE INVENTION

SA is a key component in the signal transduction pathway(s) leading to activation of certain defense responses in plants after pathogen attack. Previous studies have identified several proteins, including catalase and ascorbate peroxidase, through which the SA signal might act. A novel nucleic acid molecule encoding the SA-binding protein 2, SABP2, is described herein. SABP2 was identified through the use of a high specific activity ligand, [^{3}H]-SA (15-30 Ci/mmole). SABP2, which

is soluble, differs significantly from a previously-described SABP, which was subsequently shown to be a catalase. For example, SABP2 is much less abundant in leaf tissue than the catalase, and has an apparent native mass (M_r) of between about 20 and 40 kDa, as compared with the native molecular mass of catalase, which is approximately 240 kDa. The SABP2 from tobacco reversibly binds SA with an apparent K_d of approximately 90 nM, an affinity that is 100-150 fold higher than that between SA and tobacco catalase. Strikingly, SABP2, isolated from plant extracts, exhibits an even greater affinity (approximately 15-fold) for the synthetic functional SA analog, benzothiadiazole (BTH), which is a more effective inducer than SA of PR gene expression and disease resistance in monocots as well as dicots (Görlach, et al., 1996; Lawton et al., 1996; Morris et al, 1998; Benhamou and Bélanger, 1998).

SABP2 preferentially binds SA and active analogs that induce PR gene expression and enhanced disease resistance. Exemplary analogs include without limitation 5-chlorosalicylic acid (5-CSA) and 2,6-dihydroxybenzoic acid 2,6-DHBA). SABP2 shows only minimal binding to inactive analogs such as 4-hydroxybenzoic acid (4-HBA). See Table IV. Unexpectedly, the recombinant, purified SABP2 did not bind BTH. This likely reflects the fact that synthetic BTH is an S-methyl ester which is converted by plant esterases into the active acid form (Kaji, M., et al., 1997; Ishii, H., et al., 1999; Pillonel, C. 2001). Presumably esterases in the partially purified SABP2 preparations convert BTH to its active form before it is bound by SABP2, while such esterases are not present in the purified SABP2 produced in E. coli.

The novel SABP2 encoding nucleic acid molecules of

the invention may be used to advantage for expression of SABP2 in large quantities. They may also be used to generate transgenic plants having altered expression levels of SABP2. Such transgenic plants may be genetically engineered to overexpress or underexpress SABP2. Alternatively, knockout plants may be created wherein endogenous SABP2 gene expression is abolished. In yet another aspect, plant expression vectors can be generated wherein expression of SABP2 is under the control of an inducible promoter.

In yet another embodiment of the invention, methods are provided for identifying agents or compounds which have binding affinity for SABP2. Such agents may be used in methods for controlled modulation of plant defense responses. Exemplary agents may alter SABP2 enzymatic activity (e.g. stimulate or inhibit) and/or bind tightly to SABP2 and compete for SA binding.

Although the SABP2 encoding nucleic acid from tobacco is described and exemplified herein, this invention is intended to encompass nucleic acid molecules from other plant species that are sufficiently similar to be used interchangeably with tobacco SABP2 encoding nucleic acids for the purposes described below. For example, *Arabidopsis*, like tobacco, have a SABP2-like activity (Du and Klessig, 1997). Arabidopsis contains 18 full-length homologs of SABP2, two of which (AtSB2L5 and AtSB2L9) bind SA (Table IV) and appear to be functional equivalents (orthologs) of SABP2. Thus, using the primers described herein, one may successfully isolate and utilize SABP2 homologs and possible orthologs from other plant species using the methods provided herein.

Finally, given the homology of human nucleic acids encoding proteins having function comparable to SABP2, identification and characterization of such molecules is

encompassed within the present invention. For example, lecithin (phosphatidylcholine) cholesterol acetyl transferase (LCAT, GenBank Accession No. XO4981; SEQ ID NO: 31), an enzyme found in mammals, including humans, may also be regulated by SA and analogs thereof.

Exemplary methods for assessing the activity of molecules such as LCAT include SA-binding assays, such as the assay set forth in Example II herein. Additionally, SA or aspirin's effect on the enzymatic activity of human LCAT can be determined by pre-treating purified LCAT protein or extracts containing LCAT with 0.001mM- 1mM of SA or aspirin for 30-180 minutes at 22-37° C before adding this pre-treated protein to the standard assay for LCAT esterase activity described by Nakayama et al., 1984. The concentration of SA or aspirin in the pre-treatment will be maintained in the enzyme assay reaction.

It is known that aspirin (acetyl SA: ASA) and SA reduces the risk of heart disease and stroke. It is also believed that this protective effect of aspirin/SA is due to its ability to inhibit the aggregation of neutrophils and platelets (Weissman et al., 1991; Abramson et al., 1985). However, it is also likely that aspirin/SA exerts its protective effects by stimulating the activity of LCAT. The finding that the critical SA-binding protein for plant disease resistance responses, namely SABP2, shares both esterase/lipase activity and sequence homology with LCAT, as well as membership in the same super family of α/β fold hydrolases suggests that aspirin/SA binds LCATs. Furthermore, SA and aspirin were shown to elevate LCAT activity and reduce serum cholesterol in rats (Nakayama et al. 1984) while over expression of LCAT in rabbits (Hoeg et al., 1996) or overexpression of the combination of LCAT and cholesterol

ester transfer protein in mice (Foeger et al., 1999) were shown to protect these transgenic mammals against atherosclerosis. Therefore, it is suspected that aspirin/SA bind mammalian (e.g. human) LCATs and thereby enhance their enzymatic activity. This enhanced activity reduces the risk of atherosclerosis and associated heart disease and strokes. Thus, in accordance with the present invention, LCAT encoding sequences will also be assessed for SA binding function and enzymatic activity.

I. Definitions

The following definitions are provided to facilitate an understanding of the present invention:

The term "SABP2 function" is used herein to refer to any SABP2 activity, including without limitation expression levels of SABP2, enzymatic activity, and salicylic acid or analog binding.

An SABP2 homolog is any protein or DNA encoding the same which has similar structural properties (such as sequence identity and folding) to SABP2. An SABP2 ortholog is any protein or DNA encoding the same which has similar structural properties, and similar function (such as expression, enzymatic activity, and binding) to SABP2.

The term "pathogen-inoculated" refers to the inoculation of a plant with a pathogen.

The term "disease defense response" refers to a change in metabolism, biosynthetic activity or gene expression that enhances a plant's ability to suppress the replication and spread of a microbial pathogen (i.e., to resist the microbial pathogen). Examples of plant disease defense responses include, but are not limited to, production of low molecular weight compounds with antimicrobial activity (referred to as phytoalexins) and

induction of expression of defense (or defense-related) genes, whose products include, for example, peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes, pathogenesis-related (PR) proteins and phytoalexin biosynthetic enzymes, such as phenylalanine ammonia lyase and chalcone synthase (Dempsey and Klessig, 1995; Dempsey et al., 1999). Such defense responses appear to be induced in plants by several signal transduction pathways involving secondary defense signaling molecules produced in plants. Certain of these defense response pathways are SA dependent, while others are partially SA dependent and still others are SA independent. Agents that induce disease defense responses in plants include, but are not limited to: (1) microbial pathogens, such as fungi, oomycetes, bacteria and viruses; (2) microbial components and other defense response elicitors, such as proteins and protein fragments, small peptides, β-glucans, elicitins, harpins and oligosaccharides; and (3) secondary defense signaling molecules produced by the plant, such as SA, H_2O_2, ethylene, jasmonates, and nitric oxide.

The terms "defense-related genes" and "defense-related proteins" refer to genes or their encoded proteins whose expression or synthesis is associated with or induced after infection with a pathogen to which the plant is usually resistant.

A "transgenic plant" refers to a plant whose genome has been altered by the introduction of at least one heterologous nucleic acid molecule.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a

sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues). An "isolated nucleic acid" (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight of a given material (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-95% by weight of the given compound. Purity is measured by methods appropriate for the given compound

(e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

A "vector" is any vehicle to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The term "oligonucleotide," as used herein refers to sequences, primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The phrase "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to

the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and method of use. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically. The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either

single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as appropriate temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described

in US Patents 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

The term "promoter region" refers to the 5' regulatory regions of a gene (e.g., CaMV 35S promoters and/or tetracycline repressor/operator gene promoters).

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radio immunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion and the like.

The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic

acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

The term "selectable marker gene" refers to a gene that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other transcription control elements (e.g. enhancers) in an expression vector.

The term "DNA construct" refers to a genetic sequence used to transform plants and generate progeny transgenic plants. These constructs may be administered to plants in a viral or plasmid vector. Other methods of delivery such as Agrobacterium T-DNA mediated transformation and transformation using the biolistic process are also contemplated to be within the scope of the present invention. The transforming DNA may be prepared according to standard protocols such as those set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1995.

The phrase "double-stranded RNA mediated gene silencing" refers to a process whereby target gene

expression is suppressed in a plant cell via the introduction of nucleic acid constructs encoding molecules which form double-stranded RNA structures with target gene encoding mRNA which are then degraded.

The term "co-suppression" refers to a process whereby expression of a gene, which has been transformed into a cell or plant (transgene), causes silencing of the expression of endogenous genes that share sequence identity with the transgene. Silencing of the transgene also occurs.

Amino acid residues are identified in the present application according to the three-letter or one-letter abbreviations in the following Table:

TABLE I:

Amino Acid	3-letter Abbreviation	1-letter Abbreviation
L-Alanine	Ala	A
L-Arginine	Arg	R
L-Asparagine	Asn	N
L-Aspartic Acid	Asp	D
L-Cysteine	Cys	C
L-Glutamine	Gln	Q
L-Glutamic Acid	Glu	E
Glycine	Gly	G
L-Histidine	His	H
L-Isoleucine	Ile	I
L-Leucine	Leu	L
L-Methionine	Met	M
L-Phenylalanine	Phe	F
L-Proline	Pro	P
L-Serine	Ser	S

L-Threonine	Thr	T
L-Tryptophan	Trp	W
L-Tyrosine	Tyr	Y
L-Valine	Val	V
L-Lysine	Lys	K

Amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L-amino acid residue, provided the desired properties of the polypeptide are retained. All amino-acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

The term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, for example, due to incomplete purification, or the addition of stabilizers.

"Mature protein" or "mature polypeptide" shall mean a polypeptide possessing the sequence of the polypeptide after any processing events that normally occur to the polypeptide during the course of its genesis, such as proteolytic processing from a polyprotein precursor. In designating the sequence or boundaries of a mature

protein, the first amino acid of the mature protein sequence is designated as amino acid residue 1. As used herein, any amino acid residues associated with a mature protein not naturally found associated with that protein that precedes amino acid 1 are designated amino acid -1, -2, -3 and so on. For recombinant expression systems, a methionine initiator codon is often utilized for purposes of efficient translation. This methionine residue in the resulting polypeptide, as used herein, would be positioned at -1 relative to the mature SABP2 protein sequence.

A low molecular weight "peptide analog" shall mean a natural or mutant (mutated) analog of a protein, comprising a linear or discontinuous series of fragments of that protein and which may have one or more amino acids replaced with other amino acids and which has altered, enhanced or diminished biological activity when compared with the parent or nonmutated protein.

The present invention also includes active portions, fragments, derivatives and functional or non-functional mimetics of SABP2-related polypeptides, or proteins of the invention. An "active portion" of such a polypeptide means a peptide that is less than the full length polypeptide, but which retains measurable biological activity.

A "fragment" or "portion" of an SABP2-related polypeptide means a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to nine contiguous amino acids, typically at least about nine to thirteen contiguous amino acids and, most preferably, at least about twenty to thirty or more contiguous amino acids. Fragments of the SABP2-related polypeptide sequence, antigenic determinants, or epitopes are useful for eliciting immune

responses to a portion of the SABP2-related protein amino acid sequence for the effective production of immunospecific anti-SABP2 antibodies.

Different "variants" of the SABP2-related polypeptides exist in nature. These variants may be alleles characterized by differences in the nucleotide sequences of the gene coding for the protein such as homologs and orthologs, or may involve different RNA processing or post-translational modifications. The skilled person can produce variants having single or multiple amino acid substitutions, deletions, additions or replacements. These variants may include but are not limited to: (a) variants in which one or more amino acids residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the polypeptide, (c) variants in which one or more amino acids include a substituent group, and (d) variants in which the polypeptide is fused with another peptide or polypeptide such as a fusion partner, a protein tag or other chemical moiety, that may confer useful properties to the SABP2-related polypeptide, such as, for example, an epitope for an antibody, a polyhistidine sequence, a biotin moiety and the like. Other SABP2-related polypeptides of the invention include variants in which amino acid residues from one species are substituted for the corresponding residue in another species, either at the conserved or non-conserved positions. In another embodiment, amino acid residues at non-conserved positions are substituted with conservative or non-conservative residues. The techniques for obtaining these variants, including genetic (suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques are known to the person having ordinary skill in the art. To the extent

such allelic variations, analogues, fragments, derivatives, mutants, and modifications, including alternative nucleic acid processing forms and alternative post-translational modification forms, result in derivatives of the SABP2-related polypeptide that retain any of the functions of the SABP2-related polypeptide, they are included within the scope of this invention.

The term "functional" as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

The term "tag," "tag sequence" or "protein tag" refers to a chemical moiety, either a nucleotide, oligonucleotide, polynucleotide or an amino acid, peptide or protein or other chemical, that when added to another sequence, provides additional utility or confers useful properties, particularly in the detection or isolation, of that sequence. Thus, for example, a homopolymer nucleic acid sequence or a nucleic acid sequence complementary to a capture oligonucleotide may be added to a primer or probe sequence to facilitate the subsequent isolation of an extension product or hybridized product. In the case of protein tags, histidine residues (e.g., 4 to 8 consecutive histidine residues) may be added to either the amino- or carboxy-terminus of a protein to facilitate protein isolation by chelating metal chromatography. Alternatively, amino acid sequences, peptides, proteins

or fusion partners representing epitopes or binding determinants reactive with specific antibody molecules or other molecules (e.g., flag epitope, c-myc epitope, transmembrane epitope of the influenza A virus hemaglutinin protein, protein A, cellulose binding domain, calmodulin binding protein, maltose binding protein, chitin binding domain, glutathione S-transferase, and the like) may be added to proteins to facilitate protein isolation by procedures such as affinity or immunoaffinity chromatography. Chemical tag moieties include such molecules as biotin, which may be added to either nucleic acids or proteins and facilitates isolation or detection by interaction with avidin reagents, and the like. Numerous other tag moieties are known to, and can be envisioned by the trained artisan, and are contemplated to be within the scope of this definition.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth *in vitro* for many generations.

An "immune response" signifies any reaction produced by an antigen, such as a viral or plant antigen, in a host having a functioning immune system. Immune responses may be either humoral in nature, that is, involve production of immunoglobulins or antibodies, or cellular in nature, involving various types of B and T lymphocytes, dendritic cells, macrophages, antigen presenting cells and the like, or both. Immune responses may also involve the production or elaboration of various effector molecules such as cytokines, lymphokines and the like. Immune responses may be measured both in *in vitro*

and in various cellular or animal systems.

An "antibody" or "antibody molecule" is any immunoglobulin, including antibodies and fragments thereof, that binds to a specific antigen, such as epitopes of an SABP2 binding protein. The term includes polyclonal, monoclonal, chimeric, and bispecific antibodies. As used herein, antibody or antibody molecule contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule such as those portions known in the art as Fab, Fab', F(ab')2 and F(v).

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1997) (hereinafter "Ausubel et al.") are used.

II. Preparation of SABP2 Encoding Nucleic Acid Molecules:

Nucleic acid molecules of the invention encoding SABP2 polypeptides may be prepared by two general methods: (1) synthesis from appropriate nucleotide triphosphates, or (2) isolation from biological sources. Both methods utilize protocols well known in the art. The availability of nucleotide sequence information, such as the DNA sequences encoding SABP2, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in

the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be used directly or purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

Specific probes for identifying such sequences as the SABP2 encoding sequence may be between 15 and 40 nucleotides in length. For probes longer than those described above, the additional contiguous nucleotides are provided within SEQ ID NO: 1.

Additionally, cDNA or genomic clones having homology with SABP2 may be isolated from other species using oligonucleotide probes corresponding to predetermined sequences within the SABP2 nucleic acids of the invention. Such homologous sequences encoding SABP2 may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989), using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989) is as follows:

T_m = 81.5°C + 16.6Log [Na+] + 0.41(% G+C) - 0.63 (%

formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

The nucleic acid molecules described herein include cDNA, genomic DNA, RNA, and fragments thereof which may

be single- or double-stranded. Thus, nucleic acids are provided having sequences capable of hybridizing with at least one sequence of a nucleic acid sequence, such as selected segments of the sequences encoding SABP2. Also contemplated in the scope of the present invention are methods of use for oligonucleotide probes which specifically hybridize with the DNA from the sequences encoding SABP2 under high stringency conditions. Primers capable of specifically amplifying the sequences encoding SABP2 are also provided. As mentioned previously, such oligonucleotides are useful as primers for detecting, isolating and amplifying sequences encoding SABP2.

Antisense nucleic acid molecules which may be targeted to translation initiation sites and/or splice sites to inhibit the expression of the *SABP2* gene or production of its encoded protein are also provided. Such antisense molecules are typically between 15 and 30 nucleotides in length and often span the translational start site of *SABP2* mRNA molecules. Antisense constructs may also be generated which contain the entire *SABP2* cDNA in reverse orientation. Alternatively, the *SABP2* gene may be silenced using a construct that contains both sense and complementary antisense sequences separated by an intron sequence. This "intron-spliced hairpin RNA" approach results in total silencing of the targeted gene by forming double stranded RNA (dsRNA) structures which are degraded by the host cell machinery (Smith et al., 2000; Wesley et al., 2001).

Also provided in accordance with the present invention are transgenic plants containing the aforementioned SABP2-encoding nucleic acids, or fragments or derivatives thereof. Such transgenic plants exhibiting enhanced disease resistance are described in greater detail below.

III. Preparation of SABP2 Proteins and Antibodies:

The SABP2 protein of the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., plant cells or tissues as described in detail in Example 1. Example 1 describes the isolation of SABP2 from tobacco leaves, followed by purification by ammonium sulfate fractionation, ion-exchange, hydrophobic-interaction, and gel filtration chromatography.

Once nucleic acid molecules encoding SABP2 have been obtained, the SABP2 protein can be produced using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 vector for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

According to a preferred embodiment, larger quantities of SABP2 may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences,

translation control sequences and, optionally, enhancer sequences.

The SABP2 protein produced by gene expression in a recombinant procaryotic or eucaryotic system may be purified according to methods known in the art. In a preferred embodiment, the recombinant protein contains several (e.g., 6-8) histidine residues on the amino or carboxyl termini, which allows the protein to be affinity purified on a nickel column. If histidine tag-vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

SABP2 protein, prepared by the aforementioned methods, may be analyzed according to standard procedures. Methods for analyzing the physical characteristics and biological activity of SABP2 are set forth in United States Patent No. 6,136,552, the disclosure of which is incorporated by reference herein.

The present invention also provides antibodies capable of immunospecifically binding to proteins of the invention. Polyclonal or monoclonal antibodies directed toward SABP2 may be prepared according to standard methods. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. In a preferred embodiment, antibodies are prepared, which react immunospecifically with various epitopes of SABP2.

Polyclonal or monoclonal antibodies that immunospecifically interact with SABP2 may be utilized for identifying and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically

interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.

IV. Uses of SABP2 Nucleic Acid Molecules and Proteins:

A. Nucleic Acids Encoding SABP2-related Proteins

Nucleic acids encoding SABP2 proteins may be used for a variety of purposes in accordance with the present invention. DNA, RNA, or fragments thereof encoding SABP2 proteins may be used as probes to detect the presence of and/or expression of such genes. Methods in which nucleic acids encoding SABP2 proteins may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) Northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

The nucleic acids of the invention may also be utilized as probes to identify related genes from other plant species, animals and microbes. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, nucleic acids encoding SABP2 proteins may be used to advantage to identify and characterize other genes of varying degrees of relation to the genes of the invention thereby enabling further characterization of the molecular mechanisms controlling SA-mediated disease response in higher plants. Additionally, the nucleic acids of the invention may be used to identify genes encoding proteins that interact with SABP2 (e.g., by the "interaction trap" technique), which should further accelerate identification of the molecular components involved in the SA-mediated disease response. A new

yeast two-hybrid screen (Cytotrap) available from Stratagene, which is based on the SOS-Ras signaling pathway, is complementary to the Gal4 or LexA interaction trap system which identifies protein-protein interactions in the nucleus. In contrast, the Stratagene Cytotrap system monitors interaction in the cytoplasm, hence its name. This system, which appears to have several advantages, may be the preferred screen for SABP2-interacting proteins, particularly if SABP2 is tethered to the plasma membrane via myristoylation.

Nucleic acid molecules, or fragments thereof, encoding SABP2 genes, for example, may also be utilized to control the production of SABP2 proteins, thereby regulating the amount of protein available to participate in the induction or maintenance of disease resistance in plants. As mentioned above, antisense oligonucleotides corresponding to essential processing sites in SABP2-related mRNA molecules or other gene silencing approaches may be utilized to inhibit SABP2 protein production in targeted cells. Yet another approach entails the use of double-stranded RNA mediated gene silencing. Alterations in the physiological amount of SABP2 proteins may dramatically affect the activity of other protein factors involved in the induction or maintenance of disease resistance.

The nucleic acid molecules of the invention may also be used to advantage to identify mutations in SABP2 encoding nucleic acids from plant samples. Nucleic acids may be isolated from plant samples and contacted with the sequences of the invention under conditions where hybridization occurs between sequences of sufficient complementarity. Such duplexes may then be assessed for the presence of mismatched DNA. Mismatches may be due to the presence of a point mutation, insertion or deletion

of nucleotide molecules. Detection of such mismatches may be performed using methods well known to those of skill in the art.

Nucleic acids encoding the SABP2 proteins of the invention may also be introduced into host cells. In a preferred embodiment, plant cells are provided which comprise an SABP2 protein encoding nucleic acid such as SEQ ID NO: 1 or a variant thereof. Host cells contemplated for use include, but are not limited to, tobacco, *Arabidopsis*, rice, maize, wheat, tomato, potato, barley, canola, bacteria, yeast, insect and other animal cells including human cells. The nucleic acids may be operably linked to appropriate regulatory expression elements suitable for the particular host cell to be utilized. Methods for introducing nucleic acids into host cells are well known in the art. Such methods include, but are not limited to, transfection, transformation, calcium phosphate precipitation, electroporation, lipofection and biolistic methods.

The host cells described above or extracts prepared from them containing SABP2 may be used as screening tools to identify compounds which modulate SABP2 protein function. Modulation of SABP2 activity, for example, may be assessed by measuring alterations in SABP2-SA binding activities, SABP2 enzymatic activities, or SABP2 expression levels in the presence and absence of a test compound. Test compounds may also be assessed for the induction and/or suppression of expression of genes regulated by SABP2 proteins.

The availability of SABP2 protein encoding nucleic acids enables the production of plant species carrying part or all of an SABP2-related gene or mutated sequences thereof, in single or amplified copies. Transgenic plants comprising any one of the SABP2-related sequences

described herein are contemplated for use in the present invention. Such plants provide an *in vivo* model for examining SA-mediated defense responses, and may be particularly useful in elucidating the molecular mechanisms that modulate SA-mediated defense responses. Such plants may also facilitate the identification of other endogenously expressed gene products which play a role in SA-mediated defense responses. Methods of introducing transgenes and making knockouts in plants are known to those of skill in the art.

The alterations to the SABP2-related genes envisioned herein include modifications, deletions, and substitutions. Such modifications, deletions or substitutions can result in an SABP2 having altered characteristics or functions. Alternatively, modifications and deletions can render the naturally occurring gene nonfunctional, producing a "knock out" plant. In this context, a "targeted gene" or "knock-out" is a DNA sequence introduced into the plant by way of human intervention, including but not limited to, the methods described herein. The targeted genes of the invention include DNA sequences which are designed to specifically alter SA-mediated defense responses. Substitutions of a naturally occurring gene for a gene from a second species results in a plant which produces an SABP2-related gene from the second species. Substitution of the naturally occurring gene for a gene having a mutation results in a plant with a mutated SABP2 protein. A transgenic plant carrying a SABP2 gene is generated by direct replacement of the first plant's SABP2 gene with the second SABP2 gene. These transgenic plants are valuable for use in *in vivo* assays for elucidation of molecular mechanisms associated with local and systemic defense response systems.

Methods of use for the transgenic plants of the invention are also provided herein. Knockout plants of the invention may be treated with agents that induce local or systemic plants defense responses. Such plants provide a biological system for assessing the protective role played by SABP2. For example, agents which modulate the action or expression of SABP2 proteins may be screened in studies using SABP2 knock out plants.

In another embodiment of the invention, transgenic plants are provided that have enhanced disease resistance. Such transgenic plants may have altered SABP2 activity due to the overexpression or underexpression of SABP2-related genes.

As described above, the SABP2-related protein-encoding nucleic acids are also used to advantage to produce large quantities of substantially pure proteins, or selected portions thereof.

B. SABP2 Proteins and Antibodies

SA is a physiologically active compound in plants, animals and microbes. SABP2 almost certainly plays a role in one or more of the physiological effects mediated by SA in plants (see Cutt and Klessig, 1992). SABP2, encoded by the nucleic acid molecules of the invention, can be used in biochemical assays for screening for novel, biologically active analogues of SA. As used herein, the term "SA analogue" is intended broadly to refer to functional analogues, instead of or in addition to structural analogues of SA, which can substitute for SA to induce SA-mediated disease defense responses in plants, or other SA-mediated responses (e.g., induction of the alternative oxidase gene or flowering, as described below).

As one example, assays for SA analogues may be based

on the ability of analogues to bind SABP2 or to compete with SA for binding to SABP2. A good correlation between binding to SABP2 (measured by competition with labeled SA for binding to SABP2) and biological activity of seven SA analogues for induction of defense responses to microbial pathogens has been demonstrated, as described in U.S. Patent No. 6,136,552. One such previously identified agent, BTH, a commercially available synthetic activator of plant defense responses that is more active than SA in inducing defense responses such as *PR-1* gene expression, binds SABP2 in partially purified plant extracts about 15 fold more avidly than SA (Du and Klessig, 1997). Since SABP2 is part of the signal transduction pathway leading to disease resistance, it may be possible to enhance induction of disease resistance using analogues of SA which are not readily metabolized by the plants. It has previously been demonstrated that SA is rapidly conjugated to glucose to form SA β-glucoside (Malamy et al., 1992). This glucoside is not active for induction of disease resistance. Thus a derivative, or analogue of SA that is not readily metabolized, yet binds SABP2 and stimulates the disease resistance response, would be a superior inducer of resistance.

SABP2 likely plays a role in the numerous biological processes shown to be affected by the addition of exogenous SA, including plant disease resistance, thermogenesis, floral development, and senescence (Morris et al., 2000). Thus, these processes may be affected by altering expression levels and/or characteristics of SABP2. Current technologies of genetic engineering make both readily available. In plants, altering the level of SABP2 within the organism can be readily achieved by making transgenic plants that express SABP2 gene under a strong constitutive or inducible promoter in the sense

orientation to overproduce SABP2 or in an antisense orientation or intron-spliced hairpin RNA approach to disrupt expression of endogenous SABP2 gene(s). For example, if SA and SABP2 are both involved in flower induction, then it is feasible to inhibit flowering by blocking expression of the endogenous SABP2 gene using antisense technology or by blocking function of the endogenous SABP2 by production of a dominant negative mutant form of SABP2. In addition, if SA is a signal for this (or other) process(es), but the signal is not mediated by SABP2, then overexpression of SABP2 gene could also block this process, since SABP2 may act to sequester the SA signal, given it's high affinity for SA.

Two complementary approaches can be employed to regulate SABP2 gene expression levels. The first approach involves potato virus X (PVX)- or tobacco rattle virus (TRV)-based gene silencing, pioneered by David Baulcombe's group (Ratcliff, F. et al., 1997; Ruiz, M.T. et al., 1998). The second involves construction of transgenic tobacco in which the SABP2 gene is overexpressed (sense constructs) or underexpressed (dsRNA-mediated gene silencing). Underexpression is most effectively accomplished with constructs encoding intron-spliced RNAs with a "loopless" hairpin structure (dsRNA) that then induces gene silencing (Smith et al., 2000; Wesley et al., 2001).

The PVX-based (or TRV-based) gene silencing approach, also called virus-induced gene silencing (VIGS), has several advantages. First, it is very rapid as construction of transgenic plants is not required. Second, since VIGS can be done on adult plants, it eliminates the potential problem that the silenced gene may be required for normal plant development and/or regeneration, in which case construction of the

appropriate transgenic plants might not be feasible. Since VIGS is more effective in *Nicotiana benthamiana*, most of the silencing experiments are being done in this host or N.b. transgenic lines expressing the N gene of N. tabacum or Pto of tomato.

The transgenic approach is complementary to VIGS; although slow, it allows one to look at the effect of overexpression as well as underexpression. Moreover, in addition to facilitating more complete silencing of SABP2 and its potential ortholog(s), because the lines are stably transformed, they can be readily propagated and utilized for many generations in large number. Furthermore, some of the defense responses to be tested (e.g. ion fluxes, cell death) are more readily carried out in suspension culture which can be easily prepared from the transgenic plants.

In yet another embodiment of the invention, new response systems may be developed in plants, animals and microbes. Introduction of the SABP2 gene under control of an appropriate promoter should facilitate its expression in organisms or tissues in which SABP2 is not normally expressed. These organisms or tissues could then become responsive to SA that is either generated endogenously or applied exogenously. For example, it is possible to genetically engineer the synthesis of SABP2 in specific types of plant tissue (or animal tissue) by using tissue-specific promoters to drive (control) the expression of the SABP2 gene. Some of the tissues may not normally express an endogenous copy of the SABP2 gene or may express the gene at a very low level. Increasing synthesis of SABP2 in this way may affect the host tissue by making the tissue more responsive to SA, without affecting other tissues in which the engineered gene is not expressed. For example, if the amount of SABP2 is a

limiting factor in a physiological process such as flower development, then an enhanced production of SABP2 in tissue or cells responsible for flower development could result in greater flower production (and subsequent seed production), without affecting other physiological processes. In another example, the gene encoding SABP2 can be introduced under appropriate control elements into an organism together with a second gene under the control of a promoter which contains a SA responsive element (SARE) that makes it inducible by SA. Such an SARE from the PR-2d and PR-1 genes has recently been characterized (Shah and Klessig, 1996; Lebel et al., 1998). The expression of this second gene should then be inducible by application of exogenously applied SA. This simple strategy should be feasible if the SA-SABP2 complex directly activates the SARE-containing promoter. However, if there are other components downstream of SABP2 in the signal transduction pathway, then their presence in the tissue or organism of interest will also be necessary for the system to work. Since SA is relatively innocuous in many systems, particularly animals, and several genes in plants (from which SAREs would be obtained) are highly induced by SA (>100x), this would be an excellent system for inducible high level expression of foreign genes in transformed cells, tissues, or organisms. There is precedence for transfer of inducible gene expression systems between very divergent organisms. For example, the GAL4 system found in yeast has been shown to function in both plants and animals (Ma et al, 1988; Kakidani and Ptashne, 1988).

In another embodiment of the invention, it may be advantageous to alter the binding properties of SABP2 through genetic engineering so that it recognizes and responds to novel SA analogues. For example, an SA-like

pathway could be developed including analogues of SA and modified complementary SABP2. This system would parallel the naturally occurring SA signal transduction pathway but will be based on discrete and non-competitively binding analogues. In this way, the normal SA-based cellular functions of a plant will continue undisturbed. However, an increase in newly introduced functions may be induced. The engineered plant, containing the modified complementary SABP2 and other downstream mechanisms necessary for SA-induced expression, may be activated by the application of the non-competitive SA analogue. In this embodiment, plant functions would be influenced by two discrete signal transduction systems.

The following examples are provided to illustrate particular embodiments of the invention. They are not intended to limit the scope of the invention in any way.

EXAMPLE I

PURIFICATION AND CLONING OF SABP2-ENCODING NUCLEIC ACID MOLECULES

In order to elucidate the molecular mechanism(s) that modulate SA-mediated defense responses in plants, identify novel defense-inducing molecules and construct transgenic plants with enhanced disease/stress resistance, proteins associated with SA binding activity were identified. One such protein, SABP2, has been purified and cloned as described hereinbelow.

I. Materials and Methods:

The following protocols are provided to facilitate the practice of the present invention.

Leaf extraction: Tobacco (*Nicotiana tabacum* cv Xanthi nc [NN]) plants were grown in a greenhouse at 22°C during a 16 hour light period. 7.5 kg of fully expanded leaves from 7-8 week old plants were harvested, de-ribbed, weighed and rapidly frozen in liquid nitrogen. The tissue was stored at -80°C until further use. [All subsequent steps were carried out at 4°C unless specified otherwise.] The frozen leaves (in batches of 1.5 kg each) were ground into a fine powder using a pestle mortar and homogenized in a mixer grinder with 3 vol(w/v) of Buffer A (20 mM sodium citrate, 5 mM $MgSO_4$, 1 mM EDTA, pH 6.3, 14 mM β-mercaptoethanol supplemented with 0.1 mM phenylmethylsulfonylflouride (PMSF), 1 mM benzamidine-HCl and 1.5% (w/w) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through 2 layers of muslin cloth and 2 layers of miracloth (Calbiochem). The filtrate was centrifuged at 11,000 g in a GS-3 rotor (Sorvall) for 30 minutes, and the supernatant was collected and used for further purification.

[^{3}H] Salicylic acid-binding assay: The protein extracts/fractions were mixed with 0.1 μM [^{3}H]-SA (23.6 Ci/mmol) (custom synthesized by NEN Life Science Products) in Buffer A to bring the total volume of the reaction mixture to 150 μl. The extracts were then incubated on ice for 60 minutes. Spin columns were prepared by packing Sephadex G-25 fine (Pharmacia) equilibrated with Buffer A into 1 ml disposable syringe barrels. The G-25 was packed to 1 ml and equilibrated by centrifuging at 1000g for 4 minutes in a H-1000B rotor (Sorvall) at 4°C. The entire binding mixture was loaded onto the column and centrifuged under similar conditions. Bound [^{3}H]-SA which is present in the flow through, was mixed with 4 ml scintillation cocktail (Ecolite, ICN) and

measured in a liquid scintillation counter. Appropriate controls were also assayed. Since it was found that the chloride interferes with SA binding, care was taken to avoid its use in any form in the purification process.

Protein determination: Identification of protein in the fractions was determined using Bradford reagent (BioRad). BSA was used as standard protein.

Ammonium sulfate fractionation: Powdered ammonium sulfate [$(NH_4)_2SO_4$] was slowly added to a final concentration of 50% saturation to a continuously stirring sample of leaf extract supernatant. The slurry was incubated for an additional 30 minutes after the final addition of $(NH_4)_2SO_4$ followed by centrifugation at 11,000g in a GS-3 rotor (Sorvall) for 40 minutes at 4°C. The supernatant was brought to 75% saturation by slowly adding additional $(NH_4)_2SO_4$. After complete dissolution of the $(NH_4)_2SO_4$, the slurry was incubated for additional 30 minutes followed by centrifugation at 11,000g for 30 minutes. The pellet was resuspended in a minimum volume of Buffer A and retained for further purification.

Sephadex G-100 chromatography: Sephadex G-100 was equilibrated in Buffer A and packed into a 500 ml (C26/100, Pharmacia) column. The resuspended $(NH_4)_2SO_4$ pellet was loaded on to this Sephadex G-100 column and eluted overnight in Buffer A. Fractions of 7 ml were collected and assayed for SA-binding activity. The fractions with high [^{3}H]-SA binding activity were pooled together and precipitated with 75% $(NH_4)_2SO_4$ saturation. Further chromatographic steps were carried out using Fast Protein Liquid Chromatography system (FPLC, Pharmacia).

Q Sepharose anion exchange chromatography: The Sephadex G-100 purified $(NH_4)_2SO_4$ pellet from two, 1.5 kg preparations were resuspended in Buffer B (10 mM Bicine, pH 8.5, 14 mM β-mercaptoethanol, 0.1 mM PMSF, and 1 mM benzamidine-HCl) and desalted on a desalting column (HiPrep, 26/10, Pharmacia) equilibrated in Buffer B. The desalted protein preparation was loaded at flow rate of 1 ml/min on to a 25 ml Q Sepharose Fast Flow column (XK16/20, Pharmacia) pre-equilibrated with Buffer B. The loaded column was washed with 400 ml of 15 mM $(NH_4)_2SO_4$ in Buffer B. The bound proteins were eluted at a flow rate of 0.5ml/min with a 125 ml x 125 ml linear gradient of 15-180mM $(NH_4)_2SO_4$ in Buffer B. Fractions of 4 ml were collected and only the fractions with high $[^3H]$-SA binding activity were pooled. Solid $(NH_4)_2SO_4$ was slowly added to a concentration of 1 M to the pooled fractions for further purification by Butyl Sepharose chromatography.

Butyl Sepharose: The total pooled protein fractions (from 7.5 kg) from Q Sepharose chromatography containing 1 M$(NH_4)_2SO_4$ was applied at a flow rate of 0.5ml/min to a 15 ml Butyl Sepharose 4 Fast Flow column (XK16/20, Pharmacia) pre-equilibrated with 1.2 M $(NH_4)_2SO_4$ in Buffer B. After washing with 150 ml of 1 M $(NH_4)_2SO_4$ in Buffer B, the bound proteins were fractionated with a 75 ml x 75 ml linear gradient of decreasing $(NH_4)_2SO_4$ (1 to 0 M) in Buffer B. The 4 ml fractions were collected and assayed for $[^3H]$-SA binding. The fractions with high binding activity were pooled and precipitated with 75% $(NH_4)_2SO_4$ saturation. The pellet was resuspended in Buffer B and desalted on a desalting column (HiPrep, 26x10, Pharmacia).

Mono Q anion exchange: The desalted protein preparations from Butyl Sepharose were applied to a Mono Q HR column (5/5, Pharmacia) at a flow rate of 0.25ml/min. After washing with 20 ml of 15 mM $(NH_4)_2SO_4$ in Buffer B, the bound proteins were eluted with a 10 ml x 10 ml linear gradient of 15-180mM $(NH_4)_2SO_4$ in Buffer B at a flow rate of 0.25ml/min. Fractions of 0.5 ml were collected and fractions containing high [^{3}H]-SA binding were pooled. The protein solution was precipitated with 75% $(NH_4)_2SO_4$ saturation and desalted using a HiTrap desalting column (5ml, Pharmacia) equilibrated with Buffer C (Buffer B + 150 mM $[NH_4]_2SO_4$).

Superdex-75: The desalted protein preparation from the previous mono Q purification step was loaded at a flow rate of 0.25ml/min on to a Superdex 75 column (HR 10/30, Pharmacia), pre-equilibrated with Buffer C. The column was eluted with 25 ml Buffer C. Fractions of 0.25 ml were collected and the fractions containing high [^{3}H]-SA binding activity were pooled. The pooled fractions were precipitated with 75% $(NH_4)_2SO_4$ and desalted using a HiTrap desalting column (5ml, Pharmacia) in Buffer B.

Mono Q anion exchange: The desalted protein preparations from the Superdex 75 purification step were applied to a second mono Q column. After washing with 20 ml of 15 mM $(NH_4)_2SO_4$ in Buffer B, the bound proteins were eluted with a 10 ml x 10 ml linear gradient of 15-180 mM $(NH_4)_2SO_4$ in Buffer B. Fractions of 0.5 ml were collected and assayed for [^{3}H]-SA binding activity.

Preparative Polyacrylamide Gel Electrophoresis: The fractions with high binding activity were desalted individually with NAP-5 columns (Pharmacia) pre-

equilibrated with 5 mM Bicine, pH 8.5. The samples were concentrated by speed-vac, and then electrophoresed on a 12.5% SDS-polyacrylamide gel (16 cm x 20 cm, BioRad) as described by Laemmli (1970). After staining with 0.1% coomassie blue R-250 and destaining, the protein bands which co-purified with SA-binding activity, were cut out and sequenced at the Cornell University Protein Sequencing Facility.

Protein Sequencing: The protein samples were subjected to in-gel digestion with trypsin, and the tryptic fragments were eluted and subjected to matrix-assisted laser desorption ionization (MALDI) mass spectrometry or further purification by reverse phase HPLC. Several HPLC peaks were subjected to sequencing by Edman degradation.

Estimation of Apparent Molecular Mass: The apparent molecular mass of the native SABP2 protein was estimated by means of gel-filtration chromatography on a Superdex 75 column. The purified SABP2 (0.2 ml) from a mono Q column was loaded onto the Superdex 75 column at a flow rate of 0.25 ml/min and eluted at the same flow rate with Buffer C. The column was calibrated with the following standards: ovalbumin (43kDa), chymotrypsinogen A (25kDa) and ribonuclease A (13.7kDa) (Pharmacia).

Cloning: Based on the amino acid sequence from the SABP2 protein, several degenerate oligonucleotides, both in forward and reverse directions were custom synthesized (Invitrogen) using the preferred codon usage for tobacco. Total RNA was isolated from the young tobacco leaves using Trizol (Invitrogen) following the manufacturer's instructions. The total RNA was treated with RNase-free DNase (Promega) to remove any contaminating genomic DNA.

This purified sample of RNA was used to reverse transcribe cDNA using Superscript II Reverse Transcriptase (Invitrogen) and universal oligo-$dT_{(14)}$ with an adapter sequence.

A combination of degenerate primers (5'-ACWCARTTYTTRCCHTAYGG-3' (SEQ ID NO: 7; where W is A or T; R is A or G; Y is C or T; and H is A, C or T) encoding the sequence TQFLPYG (SEQ ID NO: 8) of peptides pk32, 34 and 36 and universal adapter primer (5'-GACTCGAGTCGACATCGA-3'; SEQ ID NO: 9) were used to PCR amplify part of the SABP2 cDNA. A small fraction of the PCR amplification products were used as a template for a second round of PCR amplification using the same set of primers. The amplified products were fractionated on a 1.5% agarose gel using 1 x TAE buffer. The DNA bands were visualized by ethidium bromide staining. The amplified DNA bands were excised out of the gel and the DNA was purified using a gel extraction kit (Qiagen). The purified DNA was then ligated into a PCR cloning vector pGEMT (Promega). DNA was prepared from the clones containing the inserts. The purified DNA was sequenced by cycle sequencing using the BigDye Terminator and analyzed on a Perkin Elmer Automatic DNA sequencer.

To isolate the 5' half of the SABP2 gene, 5' RACE ready cDNA was prepared from the total RNA isolated from tobacco leaves using the SMART RACE cDNA amplification kit (Clontech). PCR amplification was first performed with the SABP2 specific primer, E-6 (5'-AGAGATCAGTTGTATTTATG-3'; SEQ ID NO: 10) and the Universal Primer Mix (Clontech) using the 5' RACE ready cDNA as template. A second PCR amplification was performed using the E-6 primer and the Nested Universal Primer (NUP, Clontech). The amplified products were analyzed on a 1.2% agarose gel and purified as described

earlier. The fragments were then cloned into pGEMT vectors and sequenced. All PCR amplifications were carried out using the Advantage 2 PCR kit (Clontech) following manufacturer's instructions.

Expression of Recombinant SABP2 in E. coli. Full-length tobacco SABP2 was PCR amplified to introduce BamH1 enzyme sites at both ends using the primers F2 (CGCGGATCCATGAAGGAAGGAAAACACTTTG) (SEQ ID NO:51) and F3 (GCGGGATCCAGATCAGTTGTATTTATGGGC) (SEQ ID NO:52). The amplified product was cloned into the BamH1 site of pET28a (Novagen) and sequenced. Recombinant SABP2 (rSABP2) was synthesized as a soluble protein in E. coli strain BL21 (DE3) and affinity purified by Ni-NTA agarose chromatography (Novagen) as described by the manufacturer. rSABP2 was further purified on a Mono Q column as described earlier.

II. Results:

A. SABP2 Purification:

The initial attempts to purify SABP2 from tobacco leaves indicated that it is present in very low amounts. Therefore, a large amount of tissue was used as the starting material for isolation of sufficient amounts of purified SABP2 for amino acid sequencing. The soluble proteins from 7.5 kg of tobacco leaves were fractionated with ammonium sulfate, and the 50-75% saturation $(NH_4)_2SO_4$ fractions were further purified using the following purification scheme involving Sephadex G-100 gel filtration (desalting and size fractionation), Q Sepharose (anion exchanger), Butyl Sepharose (hydrophobic interaction), mono Q (strong anion exchanger), Superdex 75 (size fractionation) and a second mono Q. This scheme lead to an approximately 24,000 fold purification of SA-

binding activity (Table II). Analysis of the pooled fractions containing the SA-binding activity from the various chromatographic steps by SDS-PAGE shows the purification of SABP2 (Figure 1).

TABLE II:

PURIFICATION OF SABP2 FROM TOBACCO LEAVES

Fractionation steps	Total activity (dpm)	Percent (%)	Total protein (mg)	Specific activity (dpm mg^{-1})	Purification (fold)
Crude	7509343	100	31626.2	237	1.0
50-75%	1892720	25	4723.25	400	1.7
Sephadex G-	1385625	18.4	2216.82	625	2.6
Q Sepharose	1023300	13.6	56.925	17976	75.8
Butyl	123840	1.6	4.398	28158	118.8
Mono Q	1386700	18.4	0.368	3762930	15877.3
Superdex 75	55460	0.7	0.013	4160420	17554.5
Mono Q	45515	0.6	0.008	5689375	24005.8

The presence of SA-binding proteins was monitored using a very efficient [^{3}H]-SA binding assay. Extraction of the leaf proteins at low pH (6.3) helped in precipitating out many proteins, while the SABP2 remained soluble. Most of the non-specific SA-binding activity was removed in the 0-50% $(NH_4)_2SO_4$ fractions, and the 50-75% fraction contained most of the specific SA-binding activity (as determined by competition with unlabeled SA). Most of the high molecular weight SA-binding activity co-purifying with the carbonic anhydrase activity (e.g., SABP3, Slaymaker et al. 2002) was in the early fractions collected from the Sephadex G-100 column. The later fractions, which contained SABP2 but did not contain any detectable carbonic anhydrase activity, were

pooled and retained for further purification.

Washing of the Q Sepharose column with 400 ml of Buffer B containing 15 mM $(NH_4)_2SO_4$ removed protein lacking SA-binding activity. The presence of high concentrations of $(NH_4)_2SO_4$ in the protein fractions did not interfere with the [^{3}H]-SA binding assay.

Analysis of the SDS-polyacrylamide gel electrophoresis of the fractions from the second mono Q column indicated that three proteins (28, 30 and 32 kDa) co-purified with [^{3}H]-SA binding activity (Figure 3). The amount of the 28 kDa protein most closely tracked with level of SA binding activity, which strongly suggested that this protein was SABP2. The native molecular mass of the SABP2 was estimated to be ~28 to 30 kDa by chromatography on the Superdex 75 column (Figure 2). This estimate was in good agreement with the ~28 kDa weight estimated by SDS-PAGE. These results indicated that SABP2 is a monomeric protein. The sequences from the three proteins (28, 30 and 32 kDa) were determined and are provided in Table III, sequence identification numbers 11-27 are shown in parentheses.

TABLE III:

Amino Acid Sequences From 28, 30 and 32 kDa Proteins

Peptide	Peptide Designation	Peak#	Sequence
28kDa	Peptide #1	pk22a	VTALDLAASGTDLR(11)
"	"	pk23	XTALDLAASGTD (12)
"	"	pk55	VXALDLAASGIDLR (13)
"	Peptide #2	pk36	TPAENW/ILDTQFLPYG (14)
"	"	pk34	TPAENTLDTQF/ELM/PYG (15)
"	"	pk32	XPAENWLDTQFLPY (16)
"	Peptide #3	pk29	YPEe/nPL/gTS/tMF (17)
"	Peptide #4	pk22b	hYALFMEDLHK(18)
"	Peptide #5	pk30	AQ/KYFTDER (19)
"	"	pkx	YFQDER (20)
30kDa	Peptide #1	pk16a	LVPVDVSIDP (21)
"	Peptide #2	pk16b	i/yhfyiypIns (22)
"	Peptide #3	pk17	F/s/aLYDFVD/fG/fHK (23)
"	Peptide #4	pk25a	YVTPENNLYF (24)
"	Peptide #5	pk25b	i/sdyhis/qf/ieel (25)
32kDa	Peptide #1	pk11	NSIQPDFYANK (26)
"	Peptide #2	pk26	DIDGVPETLDLR(27)

* Sequences obtained from SABP2 purified and sequenced using a different purification scheme
"x" means any amino acid.
"/" means the amino acid at that position is either the amino acid shown before or after the '/'
Amino acids in lower case means that data was only clear enough to make a tentative assignment.

The 28 kDa protein is SABP2 because (1) it most closely co-purified with the SA-binding and (2) in an earlier purification scheme the 28 kDa protein co-purified with SA binding activity, while the presence of the 30 kDa and 32 kDa proteins were not evident in those

fractions. In addition, partial amino acid sequence analysis of tryptic peptides of the 28 kDa protein obtained in the earlier purification scheme indicated that it is the same 28 kDa protein obtained by the purification scheme described above. Further, expression of the cloned cDNA in E. coli produces a protein of the correct molecular weight and very high SA specific binding activity, with correct specificity for SA analogs (see Table IV).

B. Features of the SABP2 cDNA clone:

PCR amplification was performed using a degenerate primer (5'-ACWCARTTYTTRCCHTAYGG-3';where W is A or T; R is A or G; Y is C or T; and H is A, C or T; SEQ ID NO: 7) encoding the sequence TQFLPYG (SEQ ID NO: 8) of peptides pk32, 34 and 36 and universal adapter primer (5'-GACTCGAGTCGACATCGA-3'; SEQ ID NO: 9). The template used for PCR was the cDNA reverse transcribed from total RNA isolated from tobacco leaves. Agarose gel analysis of the PCR products did not result in any discrete bands. Thus, a second round of PCR amplification was performed using a small fraction of the PCR amplification product as the template with the same set of primers. This resulted in amplification of a ~650bp fragment which was gel purified and cloned into a pGEMT cloning vector. The clone, sequenced using plasmid specific primers, was 639 bp long with a complete 3' UTR and a polyA tail.

Analysis of the deduced amino acid sequence indicated that the clone encoded four of the five peptides (peptides #2-#5 of the 28 kDa protein in Table III) identified by partial amino acid sequence determination of SABP2. For several of these peptides, the match to the deduced amino acid sequence is close, but not exact. This may be due to sequencing errors or

may indicate that SABP2 is encoded by a gene family with the difference between peptide sequence and deduced amino acid sequence resulting from them coming from different family members.

The DNA sequence did not show significant homology to any known sequence in the NCBI database. However, the corresponding protein exhibited significant homology to several known plant proteins (Figure 5). This comparison also suggested that the cDNA clone corresponds to the C-terminal half (127 residues) of a 28-30 kDa protein.

In order to isolate the missing 5' half of the SABP2 gene, 5' RACE was carried out using tobacco cDNA as the template. The first round of 5' RACE resulted in the amplification of a very faint DNA band identified by agarose gel analysis. PCR amplification was then repeated using the E-6 (5'-AGAGATCAGTTGTATTTATG-3'; SEQ ID NO: 10) primer, a Nested Universal Primer (Clontech) and a small fraction of the first PCR product as the template. This second round of PCR amplification yielded a ~850 bp fragment which was further cloned and sequenced.

Analysis of the sequence confirmed that the amplified product from 5'RACE contained the missing 5' portion of the *SABP2* gene as well as the portion of the gene 5' to the E-6 primer that was present in the original 3' end clone. Further analysis of the 5' RACE product, together with the original 3' end clone of the SABP2 gene, revealed that the *SABP2* mRNA is at least 1079 nucleotides in length and contains a large open reading frame that encodes a 260 amino acid protein with a calculated molecular mass of 29.3 kDa and an estimated pI of 5.45.

The sequences corresponding to all five peptides obtained from the sequencing of the tryptic fragments of

the purified SABP2 protein are underlined in the deduced amino acid sequence presented in Figure 4. This result indicates that the clone corresponds to the purified SABP2 protein. The alignment of the deduced amino acid sequence of the open reading frame along with the homologous proteins (Figure 5) indicates that the clone encompasses the full length *SABP2* cDNA.

C. Proof that the 28 KDa protein is SABP2

To rigorously establish that the 28KDa protein from plant extract was SABP2, the cloned cDNA was expressed in E. coli as His_6-tagged fusion protein and the recombinant protein was purified on a Ni column and tested for SA-binding activity (Table IV). The recombinant protein exhibited very high ^{3}H-SA binding activity, and this binding was inhibited effectively by addition of a large molar excess of unlabeled SA or it's biologically active (for induction of PR genes or enhanced disease resistance) analogs but not it's inactive analogs (e.g. 4-HBA).

TABLE IV

TOBACCO SABP2 AND TWO ARABIDOPSIS HOMOLOGS PRODUCED IN E.COLI HAVE SA-BINDING ACTIVITY WHICH IS SPECIFIC FOR SA AND ITS BIOLOGICALLY ACTIVE ANALOGS

ASSAY	Bound [^{3}H]SA SABP2	Bound [^{3}H]SA AtSB2L9*	Bound [^{3}H]SA AtSB2L5*	Bound[^{3}H]SA AtSB2L1*	Biological Activity of competitor
No competitor	140206**	16486	12927	70	--
1mM unlabeled SA	160	36	121	42	active
1mM unlabeled 5-CSA	260	42	64	38	active
1mM unlabeled 2,6-DHBA	924	1072	1742	36	active
1mM unlabeled 4-HBA	95634	3620	4757	65	inactive

*AtSB2L1 is Genbank Accession NM_127926 (SEQ ID NO:32); AtSB2L5 is Genbank Accession NM_121068 (SEQ ID NO:36); AtSB2L9 is Genbank Accession NM_119878 (SEQ ID NO:40).
**Radioactivity is measured in dpm.

III. Discussion:

SABP2 is a member of the super family of alpha/beta

fold hydrolases. This gene has not previously been identified in tobacco. However, *Arabidopsis*, which has SABP2-like activity (Du and Klessig, 1997), contains a large gene family with 18 full-length members that encode proteins with 32%-57% identity and 46-71% similarity to the tobacco SABP2. They are referred to as *A.t.*SABP2 like (AtSB2L) with AtSB2L1 having the highest percentage identity over the entire 260 a.a. of tobacco SABP2 and AtSB2L18 the lowest. See Example IV. This *Arabidopsis* family of hypothetical/putative proteins shows high homology to alpha-hydroxynitrile lyases/(S)-acetone-cyanohydrin lyases (hnl or acl) isolated from cassava *Manihot esculenta* and rubber plants (*Hevea brasilienses*)(Figure 5). This enzyme, together with a β-glycosidase, releases hydrogen cyanide (HCN) through a process termed cyanogenesis, from cyanogenic glycosides (Hickel et al., 1996). HCN acts as a repellent to herbivores including insects (Kakes, 1990; Poulton, 1990). HCN may also play a role in resistance to pathogens (Hickel et al., 1996; Murphy et al., 1999).

Chivasa and Carr (1998) have also shown that KCN induces resistance to tobacco mosaic virus (TMV) in tobacco. Potassium cyanide (KCN) also induces expression of an alternative oxidase, which Carr and coworkers suggest plays a role in resistance to viruses (movement and/or replication) such as TMV, but not to bacterial or fungal pathogens (Chivasa et al., 1997; Murphy et al., 1999).

In contrast to some plants, which release large quantities of HCN from their cyanogenic glycoside reserves upon predation (Conn, 1981), most plants, including *Arabidopsis* contain little or no cyanogenic glycosides (Wäspi, et al.,1998). Perhaps in these low or non-cyanogenic plant species, SABP2/AtSB2Ls releases

small quantities of HCN from previously undetected small reserves, which are sufficient to activate a very local response(s), perhaps through induction of an alternative oxidase.

Alternatively, SABP2 may not possess hnl activity, but may have another function. Rice, for example, which lacks readily detectable levels of cyanogenic glycosides (Wäspi, et al., 1998), contains a gene termed *Pir*7b whose expression is induced following infection with the avirulent *Pseudomonas syringae* pv. syringae. Infection with avirulent P. s. s. also induces acquired resistance to the rice blast fungus, *Pyricularia oryzae* (Smith and Métraux, 1991). *Pir*7b is homologous (35% identity, 56% similarity) to the cassava and rubber plant hnl (and to SABP2; 42% identity, 61% similarity), but has no hnl activity. Rather it exhibits esterase activity towards naphthol AS-esters (Wäspi, et al., 1998).

Interestingly, *Pir7b* contains a conserved catalytic triad of serine, aspartate and histidine and a lipase signature sequence (Wäspi, et al., 1998). The N-terminal regions of lipase/esterases, like *Pir7b*, contain the serine residue of the catalytic triad and the lipase signature sequence while its aspartate and histidine reside in the C-terminal half. The serine, aspartate and histidine residues of the triad are also present and appropriately positioned within SABP2. SABP2 also contains the lipase signature sequence. This protein appears to have a functional esterase/lipase, based on its ability to release MUF (4-Methyl umbelliferone) from MUF butyrate (Figure 6). There is considerable evidence for the involvement of lipids and lipases in cellular signaling. For example, diacylglycerol activates protein kinase C which then modulates many Ca^{2+}-dependent cellular processes (Niskizuka, 1986).

Moreover, two additional *Arabidopsis* genes encoding lipase-like proteins, *EDS1* and *PAD4*, when mutated, result in increased susceptibility to pathogens. The products of both genes are thought to function upstream of SA (Falk et. al., 1999; Zhou et. al., 1998) and may form part of a signal-amplification loop with SA (Jirage et. al., 1999). However, lipase activity has not yet been demonstrated for either protein (Falk et. al., 1999; Jirage et. al., 1999). SABP2 also has strong homology to several esterases such as the ethylene-induced esterase from *Citrus sinensis* (58% identity and 72% homology, Zhong et al., 2001) and polyneurideine aldehyde esterase from *Rauvolfia serpentina* (57% identity and 75% homology) (Dogru et al., 2000).

In addition to SABP2's activity on MUF butyrate, sequence comparisons and structural analyses suggest that this protein is a lipase. Supporting this hypothesis SABP2's N-terminal sequence contains a domain (a.a. 15-127) present in mammalian lecithin (phosphatidylcholine) cholesterol acyltransferase (LCATs) and similar proteins found in mammals, yeast, *C. elegans* and *Arabidopsis*. In mammals, LCAT, which has a role in cholesterol transport and metabolism, converts phosphatidylcholine and cholesterol to lysophosphatidylcholine and cholesteryl esters in a sequential reaction on the surface of high density lipoproteins (Jones, A., 2000). LCAT can also use additional acyl donors besides phosphatidylcholine, such as phosphatidylethanolamine, and other acyl acceptors in addition to cholesterol including water. Thus, based on its homology to LCAT and other alpha/beta fold hydrolases, SABP2 may be a phospholipase involved in lipid metabolism and signaling in plants.

Taken together, these sequence analyses strongly implicate SABP2 as a critical signaling component in SA-

mediated disease resistance, perhaps by acting as an esterase/lipase.

EXAMPLE II

ANALYSIS OF SABP2 GENE COPY NUMBER, EXPRESSION PATTERNS AND ENZYMATIC ACTIVITY

Determination of SABP2 distribution and expression patterns provides valuable insight into the mechanisms and nature of plant pathogen resistance. Accordingly, experiments were performed to assess the effects of altering SABP2 expression levels on pathogen resistance.

The following materials and methods are provided to facilitate the practice of Example II. Distribution and copy number can be determined by Southern analysis and expression of SABP2 can be determined by Northern analysis, according to the methods of Sambrook et al., 1989.

SABP2 Gene Expression, Distribution, and Copy Number

Southern hybridization can be utilized to determine the distribution and copy number of SABP2 in different plant species. Homologs in humans, mouse, Drosophila and S. cerevisiae may also be identified using sequence information in the cDNA clone set forth herein. Database analysis already indicates that homologs are present in a diverse group of plants including Arabidopsis, rice, cassava and rubber tree. The cassava and rubber tree proteins are ACLs that may play a role in cyanogenesis while the rice protein, termed PIR7b, does not have lyase activity but is an esterase. As mentioned previously, expression of Pir7b is induced, in parallel with acquired resistance to the rice blast fungus Pyricularia oryzae, by infection with avirulent P.s. pv syringae (Waspi, U.

et al., 1998).

Southern blot analysis done at both low and high stringency identified only 2-4 bands with restriction enzymes that do not have recognition sequences in the SABP2 coding region. Since tobacco is amphidiploid, this result suggests that SABP2 is encoded by a single gene (but two alleles) or by a very small gene family. Slight variation in the deduced a.a. sequence of the cloned SABP2 from the a.a. sequence obtained from the purified protein for three of the five tryptic peptides also suggests the presence of at least two genes or alleles of a single gene. In contrast, Arabidopsis contains 18 AtSBP2Ls, most of which are expressed and two have been shown to have SA-binding activity (Table IV).

Expression studies have been initiated and already indicate that the tobacco SABP2 and one of its putative Arabidopsis ortholog, AtSB2L9, are induced in plants resisting infection by viral [tobacco mosaic virus(TMV) or turnip crinkle virus (TCV)] or bacterial (P. s.) pathogens. See Figure 7. These studies can be extended to other plant-pathogen interactions, e.g. P. s. pv tabaci, and P.s. pv tomato with or without AvrPto in tomato or Pto-transformed Nicotiana benthamiana, for SABP2 and P. s. and Peronospora parasitica for AtSB2L5, AtSB2L9, and other putative orthologs in Arabidopsis. While TMV induction of SABP2 expression is SA dependent, SA (or BTH) treatment alone is insufficient for this induction, suggesting the involvement of another factor. A likely candidate is ethylene since its synthesis is induced by TMV (de Laat, A.M.M. et al., 1982) and ethylene has recently been shown to induce an esterase in citrus which is highly homologous to SABP2 and the AtSB2Ls (Zhong, G.Y. et al., 2001). Therefore, induction of SABP2 and its Arabidopsis ortholog(s) by ethylene can

be tested in a similar fashion.

A general picture of the temporal and spatial expression of the two (or more) tobacco SABP2 genes/alleles is obtained by cloning the second gene/allele and designing member/allele-specific probes (usually corresponding to the divergent 3'UTR). In situ hybridization can then be used to determine a more refined expression pattern.

Localization

A better understanding of SABP2's function is facilitated by determining its subcellular location. Subcellular location can be determined by immunofluorescence microscopy, which has been used extensively, for example, to determine the subcellular locations of other proteins, including the adenovirus DBP (Voelkerding, K.V. et al., 1986) and tobacco PR-1 proteins (Carr, J.P. et al., 1989; Dixon, D.C. et al., 1991). An alternative and complementary approach is subcellular fractionation followed by western analysis using anti-SABP2 antibodies. Subcellular fractionation techniques used to identify SABP3 (Slaymaker, D.H., et al., 2002) and SABP4 and characterize NO-sensitive aconitases (Navarre, R., et al., 2000), can be readily modified for western analysis of SABP2. High affinity antibodies (Ab) have been prepared in rabbits to native and SDS-denatured SABP2. The strongest Ab is to the native SABP2 and is highly specific, reacting only with SABP2 in western analysis of total protein extracts from mock- or TMV-infected tobacco (see Fig. 7B). Thus, this Ab is very suitable for immunofluorescence microscopy.

Initial localization is done in TMV-infected plants or in a transgenic line overexpressing SABP2. Alternatively, analysis may be conducted of plants

overexpressing SABP2 tagged at its C-terminus with the HA epitope, for which highly specific, high affinity Ab are commercially available. Others have used a similar approach to localize bacterial avr factors/effectors to the plant plasma membrane (PM) (Nimchuk, Z. et al., 2000; Shan, L., et al., 2000). Interestingly, SABP2 possesses a myristoylation site near its N-terminus (12GACHGG17; SEQ ID NO:28), in addition to potential glycosylation (114NSSF117; SEQ ID NO: 50) and PKC and CK2 phosphorylation sites. Since myristoylation frequently directs proteins to the plasma membrane and many receptors and other signaling components are associated with the plasma membrane, SABP2's putative myristoylation sequence will be mutated to determine if it is necessary for fatty acylation, membrane association or function of SABP2. AtSB2L5 and AtSB2L9, SABP2 related genes in Arabidopsis, also have myristoylation sites near their N-terminus.

Enyzmatic activity

SABP2 and its ortholog(s) possess enzymatic activity. A comparison of amino acid sequence and/or enzyme activity(s) for SABP2 and its Arabidopsis ortholog(s) (which have SA-binding activity and whose silencing suppresses certain defense responses) will facilitate the identification of SABP2's biochemical activity that is relevant to SA-mediated defense responses.

Since SABP2 contains the catalytic triad and the lipase signature sequence, the recombinant protein was tested for lipase activity. Highly purified recombinant SABP2 (rSABP2) exhibited lipase/esterase activity with 4-methylumbelliferone butyrate in an in gel assay, and with para-nitrophenyl (pNP) butyrate in a solution assay

(Figure 6A). rSABP2 also cleaved esters containing long carbon chains such as pNP palmitate (C-16) and pNP myristate (C-14) (Figure 6B), thereby demonstrating true lipase activity. Addition of SA to the reaction stimulated lipase activity 3-6 fold (Figure 6B). This stimulation required SA binding to SABP2, since it was abolished in reaction conditions that prevented SA binding. In contrast to SABP2, the lipase from Mucor meihei did not exhibit stimulation by SA, indicating that SA stimulation of lipase activity is not a general phenomenon (data not shown). The lipase activity assay was performed as described by Yang et al. with modifications to allow SA binding to SABP2. The standard 1 ml assay mixture consisted of 1 mM substrate in 50 mM Bicine pH 8.0, 0.05% Triton-X100. Following a 60 min preincubation on ice in absence or presence of 1 mM SA, the reaction was allowed to proceed at 24°C for 60 min. A 100 mM stock solution of p-NPM or p-NPP was prepared in acetonitrile. Lipase activity was estimated colorimetrically (Unicom UV1, Spectronic Unicom, UK) by measuring the liberation of para-nitrophenol from p-NPP or p-NPM at 410 nm. Measurements from control reactions without SABP2 were subtracted from each reaction. For non SA-binding conditions, Tris-HCl pH 8.0 (50 mM) was used in placed of Bicine buffer.

To further assess whether SABP2 has an additional enzymatic activity, a set of model substrates are employed in a systematic approach to identify the enzyme activity(s) of SABP2 and its Arabidopsis ortholog(s). This set has previously been employed by Wäspi et al. (Waspi, U. et al., 1998) and Baudouin et al (Baudouin et al., 1997) to characterize two other α/β fold hydrolases, Pir7b of rice and Hsr203J of tobacco, the first of which is closely related by sequence to SABP2 and the AtSB2Ls.

Like SABP2 they contain the catalytic triad and lipase signature sequence and are induced at the transcription level during defense responses to pathogens. Pir7b's preferred substrate is naphthol AS-esters while Hsr203J prefers short-chain dinitrophenyl acyl esters. For esterase activity the following potential substrates, p-nitrophenyl butyrate, acetylcholine, 2-naphthol butyrate/acetate and 2-naphthol AS-acetate are employed. Amidase activity is monitored with acetanilide and nitro-acetanilide, while epoxide hydrolase activity analysis utilizes 9R-10S-epoxystearic acid. Protease activity is assayed with casein as the substrate.

Sequence comparisons have revealed that SABP2 is closely related to several proteins with hydroxynitrile lyase (Hnl) activity; these proteins also contain the catalytic triad and a lipase signature sequence. Based on sequence homology, the AtSB2Ls also have been designated Hnls. To test if SABP2 and the AtSB2Ls with SA-binding activity exhibit Hnl activity, the decomposition of cyanohydrins is monitored as described by Selmar et al. (1987) and Hasslacher et al. (1996) in the absence or presence of SA. If SABP2 exhibits SA-stimulated Hnl activity, it is possible that SABP2 signals defenses via HCN production. In contrast to some plants that release large quantities of HCN from large cyanogenic glycoside reserves upon predation, most plants, including tobacco and Arabidopsis, contain little or no cyanogenic glycosides. Perhaps in these non-cyanogenic plant species, SABP2/AtSB2Ls release small quantities of HCN from previously undetected small reserves, and these are sufficient to activate a local response(s). Consistent with this possibility, treating tobacco with KCN induces resistance to TMV, possibly due to increased expression of alternative oxidase (Murphy et al., 1999).

The data described above (Figures 6A and 6B) indicate that SABP2 (and its Arabidopsis ortholog[s]) is a lipase, whose activity is stimulated by SA binding. SABP2's lipase activity and SA's ability to alter this activity suggests a link between SA and lipids/fatty acids. While the nature of this connection is presently unclear, it is intriguing that not only do two other proteins involved in disease resistance (EDS1 and PAD4) have a putative lipase activity and interface with SA (perhaps through a positive feedback loop), but several more recent reports provide further evidence for a role of lipids/FAs in SA-mediated defense signaling. Work on the Arabidopsis mutant ssi2 suggests that oleic acid or its derivative suppresses SA-mediated defense responses such as PR-1 induction and resistance to P.s. and P. parasitica, while in contrast it acts in conjunction with JA to induce PDF1.2 expression and resistance to Botrytis cinerea (Kachroo et al., 2001). Lamb and colleagues (Maldonado et al., 2002) recently described another Arabidopsis mutant dir1 which is defective in induction of systemic resistance. DIR1 is a putative lipid transfer protein, which is required for the production or transmission of the mobile signal that moves from infected tissue through the phloem to induce SAR in uninoculated parts of the plant.

EXAMPLE III

SABP2 IS IMPORTANT FOR RESISTANCE TO PATHOGENS

The present inventors have demonstrated that SABP2 silencing in plants gives rise to decreased resistance to TMV and also repressed induction of the PR-1 gene by TMV infection or by SA treatment.

Materials and Methods

Plasmid Construction and Plant Transformation. The RNAi-SABP2 construct was made in the pHANNIBAL vector by (Smith et al. 2000) inserting a 404 bp fragment corresponding to the 5' portion of SABP2. The fragment corresponding to the sense arm of the hairpin loop was generated using the primers F6 (CCGCTCGAGATGAAGGAAGGAAAACACTTG) (SEQ ID NO: 53) and F7 (GGGGTACCAGATCAGTTGTATTTATGGGC) (SEQ ID NO:54) and cloned in the Xho1-Kpn1 site. The fragment for the anti-sense arm was generated using the primers F2 (described above) and G2 (GCGGGATCCCTGAGTATCCAACCAATTCTCGG) (SEQ ID NO:55) and was cloned into the BamH1 site. The Not1 fragment from pHANNIBAL containing SABP2 was then subcloned into binary vector pART27. The sequences of these constructs were confirmed by DNA sequencing. Agrobacterium strain LBA4404 was transformed with the silencing construct by electroporation. Plant transformations, regeneration and maintenance of the transgenic lines were carried out as described by Shah and Klessig (Shah et al., 1996).

RNA Blot Analysis and RT-PCR Analysis. Total RNA was extracted from tobacco leaves as described by Kumar et al. (1997). Ten μg total RNA per lane was used for RNA blot analyses. Blots were hybridized with desired probes. Hybridizations were carried out as described by Tang et al. (1999) and exposed to phosphorImager screen or X-ray film.

First strand cDNA was synthesized using 2 μg total RNA isolated from control and silenced plants as described above. Semi-quantitative RT-PCR analysis was performed using 1 μl of the cDNA in a 20 μl reaction mixture containing primers G6 (TGGCCCAAAGTTCTTGGC) (SEQ ID NO: 56) and E6 (AGAGATCAGTTGTATTTATG) (SEQ ID NO:10)

which anneal outside the region used for silencing SABP2 expression. Control reactions to normalize RT-PCR amplifications were run with the primers derived from constitutively expressed translation elongation factor 1. (*EF1α*) (forward, TCACATCAACATTGTGGTCATTGGC (SEQ ID NO:57); reverse, TTGATCTGGTCAAGAGCCTCAAG (SEQ ID NO:58)). PCR was performed for 30 cycles at 55°C annealing temperature.

Results

SABP2 Expression is Required for Complete Local and Systemic Resistance to TMV. To assess the role of SABP2 in defense signaling, SABP2 expression was silenced using RNA interference (RNAi) (Wesley et al., 2001). RNA blot analysis of 16 independently generated T_1 lines expressing the RNAi-SABP2 construct revealed that SABP2 expression was suppressed more than 75% as compared with the empty vector control plants (Fig. 8A). These 16 lines develop TMV lesions which on average were 41% larger than on the empty vector lines (Fig. 8B), as predicted if SABP2 plays a positive role in resisting TMV infection. Note that in the SA-deficient, NahG transgenic tobacco lines TMV lesion size was on average 23% larger (Gaffney, T. et al., 1993). Thus, suppression of SABP2 expression appears to be at least as disruptive to TMV resistance as destruction of the SA signal.

RNA blot analysis of 5 independent T_2 lines similarly revealed little SABP2 transcript accumulation before or after TMV infection, and the lesions were on average 34% larger than those on the control lines (Figure 8E). Moreover, transcripts for the TMV coat protein (CP) accumulated to higher levels in the inoculated leaves of SABP2-suppressed lines as compared

with control plants.

SA induction of PR-1 expression, which is associated with local resistance to TMV, was also affected in SABP2-silenced plants. Suppression of SA-induced PR-1 expression was readily detected in the T_1 generation of the five lines SABP2-silenced plants in which little, if any, SABP2 transcripts could be detected by RT-PCR under the conditions used (Figures 8B and 8F). In the T_2 generation of these five lines the level of SABP2-silencing was more variable (Figure 8G). In plants in which SABP2 transcript was undetectable SA induction of PR-1 expression was suppressed (see Figure 8G, e.g. transgenic 1-2). However, in plants in which silencing was less effective, suppression of PR-1 induction was poor (e.g. transgenic 1-3 and 1-4). These results suggest that silencing was less effective in the T_2 generation and that suppression of SA-induction of PR-1 expression was dependent on the level of SABP2 silencing.

Whether SAR development also is suppressed in the SABP2-silenced lines was then assessed. In control plants, the lesions formed after a secondary infection were ~50% smaller than those produced after a primary infection (Figure 9A and 9B); this reduction in secondary lesion size is a common marker for SAR. By contrast, the lesions formed on secondarily inoculated SABP2-silenced plants were as large as those formed after a primary infection and 2.5-fold larger than those exhibited by control plants. SABP2-silenced plants also exhibited increased viral replication, as indicated by higher levels of TMV movement protein (MP) transcript in the systemic leaves of SABP2-silenced plants than control plants after secondary inoculation (Figure 9C). In addition, systemic expression of the PR-1 gene, another common marker for SAR, was reduced in SABP2-silenced

plants. Unlike control plants, whose uninoculated leaves accumulated low to moderate levels of PR-1 transcript following a primary infection (Figure 9C), the systemic leaves of SABP2-silenced plants contained little to no PR-1 mRNA. Following a secondary infection with TMV, however, the challenge-inoculated leaves of SABP2-silenced plants accumulated more PR-1 transcripts than those of control plants. Taken together, these results suggest that SABP2 plays a role(s) in restricting viral replication/spread in TMV-inoculated leaves, as evidenced by increased lesion size and greater accumulation of transcripts for TMV CP. They also indicate that SABP2 expression is required for systemic PR-1 expression and the characteristic reduction in lesion size and viral replication associated with SAR.

Discussion

The combined observations that SABP2 binds SA with high affinity, is present in exceedingly low concentrations, and displays SA-stimulated enzymatic activity, provide evidence that SABP2 is a receptor for SA. Further supporting this hypothesis, local and systemic resistance in SABP2-silenced plants was disrupted at least as effectively as in SA-deficient tobacco expressing the nahG transgene. Following a primary infection with TMV, the lesions formed on T_1 and T_2 generations of SABP2-silenced plants were on average 41% and 34% larger, respectively, than those of control plants, while those formed on NahG tobacco were only 23% larger on average (Gaffney et al. 1993). In addition, neither SABP2-silenced nor nahG-expressing plants developed SAR. Further evidence that SABP2 is an SA receptor is the reduced ability of SA to induce PR-1 expression in plants effectively silenced for SABP2.

Interestingly SABP2 silencing was more variable and generally less effective in T_2 versus T_1 plants. Less effective silencing of SABP2 correlated with poor suppression of PR-1 induction, suggesting that the residual SABP2 level in these T_2 plants is at or above a threshold required for SA induction of PR-1, while in the T_1 plants and a minority of T_2 plants (e.g. transgenic 1-2 in Figure 8G), it is below this level. Since local and systemic resistance were impaired in all T_2 plants tested, the level of SABP2 required for resistance appears to be higher than that needed for PR-1 gene activation. The efficiency of SABP2 silencing did appear to influence how severely resistance was impaired, however, because the primary TMV lesions on T_2 plants were not as large as those on T_1 plants.

The reduction in local resistance, inability to activate SAR and loss of SA responsiveness exhibited by SABP2-silenced plants is very similar to the phenotype of SA-insensitive, SAR-defective npr1/nim1/sai1 Arabidopsis mutants (Cao et al., 1994; Delaney et al., 1995; Glazebrook, 1996; Shah, 1997). NPR1 is an important signal transducer that functions downstream of SA in the defense signaling pathway. This protein contains ankyrin repeats and shares limited homology with the IκBα subclass of transcription factors/inhibitors in animals, which regulate immune and inflammatory responses (Cao et al., 1997; Ryals, 1997). Recent studies have revealed that NPR1 is maintained in the cytoplasm as an oligomer formed through intermolecular disulfide bonds (Mou et al. 2003). Treatment with SA (or its analog 2,6-dichloro isonicotinic acid) or infection with pathogens alters the cellular reduction potential, thereby promoting monomerization of NPR1; these monomers then are translocated to the nucleus, a prerequisite for PR-1 gene

activation (Mou et al., 2003; Kinkema et al. 2000). While these findings provide one mechanism of action for SA, an additional mechanism(s) also must exist to account for the poorer induction of PR-1 expression and disease resistance in transgenic npr1-1 mutant Arabidopsis that constitutively accumulate monomeric, nuclear-localized NPR1 than in 2,6-dichloro isonicotinic acid-treated wt plants (Mou et al., 2003). Furthermore, NPR1 was recently shown to regulate SA-mediated suppression of jasmonic acid signaling via a mechanism that does not require nuclear localization (Spoel et al., 2003). Thus the similarities between SABP2-silenced and npr1/nim1/sai1 plants strongly argue that SABP2 is an important component of this pathway that functions at a point downstream of SA.

The discovery that SABP2 displays SA-stimulated lipase activity and SABP2 is required for local and systemic resistance, suggests SABP2's lipase is required to signal resistance. One possible mechanism for resistance-specific SABP2 activation is via direct stimulation of its lipase activity by SA. This might be mediated by SA-facilitated displacement of the lid, a surface loop found on many lipases and other α/β fold hydrolases that covers the active site and regulates substrate selection and binding (Nardini et al., 1999). SABP2 activity also may be increased by enhanced gene expression since SABP2 transcript levels increased in TMV-infected tobacco plants (Figure 8E).

The mechanism through which SABP2's lipase activity transduces the defense signal is not known. However, there is growing evidence that lipids play an important role in signaling disease resistance. The EDS1 and PAD4 proteins of Arabidopsis, which are putative lipases, are required to transduce the resistance signal following

pathogen recognition by a specific class of resistance (R) genes. While these proteins share little homology with SABP2, all three contain the catalytic triad and the lipase signature sequence (Falk et al., 1999; Jirage et al., 1999). A fatty acid (FA) desaturase also has been linked with resistance signaling. The ssi2 mutation in Arabidopsis, which impairs stearoyl desaturase activity and thereby alters cellular FA content, confers constitutive activation of several SA-associated defense responses and suppression of certain JA-dependent defenses (Kachroo et al., 2001; Shah et al., 2001). More recently, a defect in a putative apoplastic lipid transfer protein caused by the dir1-1 mutation was shown to impair systemic, but not local, resistance in pathogen-infected Arabidopsis (Maldonado et al., 2002). DIR1 therefore appears to play a role in generating or translocating the SAR signal that moves from inoculated leaves to other parts of the plant. Given that both dir1-1 mutant and SABP2-silenced plants are defective in developing SAR, it is tempting to speculate that SABP2's SA-stimulated lipase activity generates a SAR-inducing lipid (or lipid derivative) that is translocated by the DIR1-encoded lipid transfer protein to the uninoculated parts of the plant. Since some AtSB2L (also referred to as SABP2L) proteins do not bind SA, these may bind other ligands, such as stress-associated hormones like JA or abscisic acid. Indeed, these proteins may comprise a family of receptors that, upon binding their cognate ligand, exhibit enhanced hydrolase (lipase/esterase) activity. Different members, or sets of members, would likely display distinct substrate specificities, thereby ensuring that the proper response is signaled. Alternatively, sequence similarity between SABP2 and other known proteins, including several plant

hydroxynitrile lyases (Hnls) and lecithin (phosphatidylcholine) cholesterol acyl transferase (Jonas et al., 2000) from animals, raises the possibility that some SABP2/AtSB2L family members have other enzymatic activities. For example, some SABP2/AtSB2L members might catalyze the release of toxic hydrogen cyanide (HCN) from cyanogenic glycosides, perhaps for defense against herbivorous insects and pathogens (Hickel et al., 1996). Tobacco and Arabidopsis contain very low to undetectable levels of cyanogenic glycosides; however, an SABP2/AtSB2L Hnl might be able to release small quantities of HCN from a previously undetected store of cyanogenic glycoside and thereby activate local defense responses. In support of this possibility, KCN treatment induces TMV resistance in tobacco, possibly by increasing expression of alternative oxidase (Chivasa et al., 1998). Alternatively, it is possible that one or more members of the SABP2/SABP2L family has JA-stimulated protease activity which releases systemin and other peptide hormones from a common precursor for systemic activation of plant defenses against herbivorous insects (Pearce et al., 2003).

In summary, the results presented herein provide evidence that SABP2 is a resistance signaling receptor for SA. The steps activated downstream of this SA effector protein are not yet known. However, the ability of SA to regulate SABP2's lipase activity suggests a mechanism through which lipids/FA are linked to the SA-dependent defense signaling pathway.

EXAMPLE IV

IDENTIFICATION AND CHARACTERIZATION OF ARABIDOPSIS SB2LS

The extensive collection of mutants affecting disease resistance and SA-mediated defense responses and the availability of its genomic sequence make Arabidopsis

an ideal plant to study disease resistance. Moreover, Arabidopsis contains an SABP2-like activity (Du, H. et al., 1997) and has a large gene family (18 full-length members) which encode proteins with 32-57% identity and 46-71% similarity to SABP2, two of which have already been shown to have specific SA-binding activity (see Table IV), one of which is induced by infection. Note that while at least 15 are expressed (ESTs available) and have sequences suggesting they are esterases, lipases or lyases, none have a known function. Thus, characterization of this family to determine which has SA-binding activity and of these, what affect does silencing (knock out (KO) or dsRNA mediated) have on the plant's phenotype, particularly with respect to disease resistance, will help further understanding of the disease resistance pathway.

Two parallel types of experimentation are performed initially. First, select members of this family continue to be cloned, expressed, and analyzed for the SA-binding. A dendrogram of SB2Ls (Fig. 10) suggests that they can be grouped into five subfamilies. To date several members of subfamily I and II, the subfamilies most closely related to tobacco SABP2, have been tested for SA-binding activity. All members of subfamilies I and II and select members of the more distant subfamilies III-V can also be analyzed.

In parallel two approaches are used to obtain transgenic or mutant Arabidopsis with altered expression of one or more members of the AtSB2L family. If a limited number have SA-binding activity, then T-DNA derived KO or activation-tagged mutants for these family members will be obtained from the UW Arabidopsis KO Facility (α and β populations) and from the Salk Institute Genomic Analysis Lab's libraries. KOs have been obtained for five AtSB2Ls

from the Salk Institute collection that correspond to two SA-binding AtSB2Ls, AtSB2L5 (subfamily I) and AtSB2L9 (subfamily II), another member of subfamily I (AtSB2L1), one member of subfamily IV (AtSB2L16), and one of subfamily V (AtSB2L14). These five KO lines can initially be screened for altered defense responses to avirulent P. s. (e.g. HR, pathogen growth) or to SA treatment (e.g. induction of PR-1, PR-2, and PR-5), as well as SA-binding activity for AtSB2L7, AtSB2L14 and AtSB2L15. (If, as anticipated, one or more of these three latter KOs do not have SA-binding activity, it will serve as a good negative control). A secondary screen of the more interesting KOs would include resistance to an avirulent pathovar of P. parasitica and expression of other defense genes such as GSTs, PR-3, PR-4 and defensin (PDF1.2).

Knockout of a single AtSB2L may not alter defense response because of possible redundancy. If there are only a small number of AtSB2Ls with SA-binding activity, then one approach will be to cross the different KO lines to generate lines with 2, 3, or more of the SA-binding AtSB2Ls silenced. This approach is less likely to be successful if a significant number (e.g. >5) of the AtSB2Ls bind SA. The better approach then will be dsRNA-mediated gene silencing in stable transgenics as outlined above for SABP2. Because of sequence divergence among the family members, particularly at the DNA level, silencing of all members with one construct is not possible. Initially and in parallel with initial screening of the readily available KOs, all members of subfamily II will be silenced with one construct and most subfamily I members using sequences that are highly conserved within that subfamily. Effective silencing requires a minimum of 50 nucleotides with 80% or more identity. In subfamily II there is a 65 nucleotide region

with 80% identity among all five members located between 678 and 742, with the A of the initiation codon of AtSB2L9 designated 1. No such conserved region is present in all four subfamily I members; however, there is a 153n region with 79% identity for AtSB2L1, 2, and 8 located between 3 and 155 with the A of AtSB2L1's ATG as 1. This sequence will be used to silence these three members in the Salk line in which the fourth member, AtSB2L5, is knocked out. Also the number of AtSB2Ls that bind SA are determined, careful analysis of sequences of these genes may identify a highly conserved region against which the dsRNA could be targeted for silencing of all, or at least most, of the SA-binding AtSB2Ls.

Construction and characterization of transgenic lines that overexpress one of the SA-binding AtSB2Ls may also be informative and will be done for a limited number of these AtSB2Ls, particularly if silencing of that AtSB2L alters defense responses. The expression of SA-binding AtSB2Ls will also be monitored to see if they are induced by infection with avirulent or virulent pathogens (e.g. TCV, P. s., P. parasitica) or treatment with SA (or BTH).

This large family of AtSB2Ls may provide an opportunity to identify a region of these proteins which is required for SA binding. Comparison of the a.a. sequences of these AtSB2Ls with SA-binding activity (and SABP2) versus those of non-binding homologues may reveal a region (and possibly even critical a.a.'s) that is likely involved in SA binding. This will be confirmed by swapping of this putative domain between closely related binding and non-binding homologues, preferably within the same subfamily. Site-directed mutagenesis could then be used to confirm or dispel the involvement of a.a.'s predicted to be important based on sequence analysis.

Once the enzymatic activity involved in transmitting the SA signal by SABP2 and its Arabidopsis ortholog(s) has been defined, then the effects of these mutations on it, as well as SA binding, will be determined.

The Arabidopsis thaliana SABP2-like gene family members are referred to herein as follows:

AtSB2L1, Genbank Accession No NM_127926 (SEQ ID NO:32),
AtSB2L2 Genbank Accession No NM_127924 (SEQ ID NO:33),
AtSB2L3 Genbank Accession No NM_127925 (SEQ ID NO:34),
AtSB2L4 Genbank Accession No NM_127922 (SEQ ID NO:35),
AtSB2L5, Genbank Accession No NM_121068 (SEQ ID NO:36),
AtSB2L6 Genbank Accession No NM_127919 (SEQ ID NO:37),
AtSB2L7 Locus At2g23560 Accession No NM_127920 (SEQ ID NO:38),
AtSB2L8 Genbank Accession No NM_127923 (SEQ ID NO:39),
AtSB2L9 Genbank Accession No NM_119878 (SEQ ID NO:40),
AtSB2L10 Genbank Accession No NM_114904 (SEQ ID NO: 41),
AtSB2L11 Genbank Accession No NM_113902 (SEQ ID NO:42),
AtSB2L12 Genbank Accession No NM_117058 (SEQ ID NO:43),
AtSB2L13 Genbank Accession No NM_102400 (SEQ ID NO:44),
AtSB2L14 Genbank Accession No NM_103121 (SEQ ID NO:45),
AtSB2L15 Genbank Accession No NM_105591(SEQ ID NO:46),
AtSB2L16 Genbank Accession No NM_117770 (SEQ ID NO:47),
AtSB2L17 Genbank Accession No NM_111924 (SEQ ID NO:48), and
AtSB2L18 Genbank Accession No NM_125216 (SEQ ID NO:49).

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.